

**Expression and function of NG2/HMPG in human cartilage.**

*Kim Suzanne Midwood*

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I hereby declare that this thesis has been composed by myself and has neither been presented nor accepted in any previous application for a degree. All work presented in this thesis was, unless acknowledged, carried out by myself. All sources of information have been acknowledged by reference.

Kim Midwood

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## ABSTRACT

NG2 is a transmembrane protein expressed by cells as both a chondroitin sulphate rich proteoglycan or a CS unmodified core protein. It is expressed by neuronal and glial cells. Recent studies have shown NG2 to be identical to human melanoma proteoglycan, which is expressed predominantly by melanoma cells. It is thought to be a cell surface receptor for extracellular matrix proteins including type VI collagen and fibronectin, as well as being involved in the response of some cells to platelet derived growth factor. It may also be a site for intracellular phosphorylation. NG2 is therefore potentially important in cell adhesion, proliferation and signal transduction in these cells. In view of the expression of NG2 by developing cartilage in rats and putative roles in cell-matrix interactions we have investigated the expression of NG2 by normal human cartilage and adult osteoarthritic cartilage and its potential to function as a cell signalling molecule.

Cryostat sections of human fetal knee joints and articular cartilage from macroscopically normal and osteoarthritic adult knee joints obtained at autopsy were immunostained with a panel of antibodies against rat NG2 and HMPG. Chondrocytes in fetal cartilage and normal adult cartilage showed strong immunoreactivity with anti-NG2/HMPG antibodies. NG2 is also present in osteoarthritic cartilage, but expression is down regulated. Western blots of whole cell lysates from macroscopically normal and OA femoral condyles, revealed a smeared component of molecular weight greater than 400 kD and a faint band at 250 kD. The 250 kD protein became predominant upon digestion with chondroitinase ABC. Autoradiographs of  $I^{125}$  labelled chondrocyte lysates which had been precipitated with anti-HMPG antibody confirmed NG2 was present in human adult articular chondrocytes. Immunofluorescence staining of cultured chondrocytes revealed that NG2 is distributed in a punctate pericellular pattern which does not appear to coincide with that of ECM proteins type II or VI collagen and FN, actin stress fibres or PDGF  $\alpha$ R. Anti-HMPG antibodies increase chondrocyte adhesion to type VI collagen and this modulation of cell adhesion can be inhibited by pertussis toxin.

These studies show that NG2 is expressed by human fetal and adult chondrocytes. In adult articular chondrocytes the core protein is chondroitin sulphated and both CS modified and CS free forms are simultaneously expressed. Expression is down regulated in OA cells. NG2 appears to influence chondrocyte interaction with type VI collagen, via a pertussis sensitive G protein. Cell matrix binding functions and cell signalling activity suggest that NG2/HMPG has important roles in the regulation of articular chondrocyte activity in health and disease.

## ABBREVIATIONS

AA	amino acid
AB	avidin-biotin
ALL	acute lymphoblastic leukaemia
AML	acute myeloid leukaemia
AP	alkaline phosphatase
APS	ammonium persulphate
BCIP	5-bromo-4-chloro-3-indolyl phosphate
BSA	bovine serum albumen
CII	type II collagen
CIV	type IV collagen
CVI	type VI collagen
cABC	chondroitinase ABC
cAMP	cyclic adenosine monophosphate
cd	cytochalasin D
cGMP	cyclic guanosine monophosphate
CNS	central nervous system
CS	chondroitin sulphate
CSPG	chondroitin sulphate proteoglycan
DAB	diamino benzidine
DAG	diacyl glycerol
DEC	diethylcarbamizine
DTT	dithiothreitol
EASY	enhanced analysis system
ECL	enhanced chemiluminescence
ECM	extracellular matrix
EDTA	ethylenediaminetetraacetic acid
Endo H	endoglycosidase H
FAK	focal adhesion kinase
FCS	fetal calf serum

FITC	fluorescein isothiocyanate
FN	fibronectin
GAG	glycosaminoglycan
gen	genistein
G protein	GTP binding protein
GTP	guanosine triphosphate
HA	hyaluronic acid
HMWC	high molecular weight component
HMPG	human melanoma proteoglycan
HPLC	high performance liquid chromatography
HRP	horse radish peroxidase
HS	heparin sulphate
IGF	insulin-like growth factor
IGF-1R	insulin-like growth factor receptor
IR	insulin receptor
IRRR	insulin receptor related receptor
KS	keratin sulphate
LN	laminin
MLL	myeloid/lymphoid leukaemia
MW	molecular weight
NBT	nitroblue tetrazolium
OA	osteoarthritic
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PDGF	platelet derived growth factor
PDGF $\alpha$ -R	platelet derived growth factor $\alpha$ receptor
PG	proteoglycan
PKC	protein kinase C
PLC	phospholipase C
PLL	poly-L-lysine
PMSF	phenyl methyl sulphonyl fluoride

pt	pertussis toxin
SDS	sodium dodecyl sulphate
st	staurosporine
TBS	tris buffered saline
TBST	TBS tween
TEMED	N'N'N'N-tetramethyl ethylene diamino
TGF- $\beta$	transforming growth factor- $\beta$
TN	tenascin
TRITC	tetramethyl rhodamine isothiocyanate

## **1.0. INTRODUCTION**

### **1.1. HUMAN ARTICULAR CARTILAGE**

#### **1.1.1. Cartilage structure and function**

Articular cartilage is a specialised connective tissue whose function is to dissipate compressive loads and protect the underlying bone. Adult cartilage is a hypocellular, hyperhydrated, avascular, aneural, alymphatic tissue. No mitotic division occurs in healthy cells. It contains a small number of cells (chondrocytes) which secrete an abundant extracellular matrix (ECM). Chondrocytes in normal cartilage maintain a balance between synthesis, secretion and deposition, and degradation and removal of matrix molecules. Collagen is the major structural component of cartilage, and provides the strength of the tissue by resisting shear and tensile forces. Type II collagen (CII) is the most abundant protein in cartilage, where it forms a fibrillar network which is found throughout the entire ECM. The minor collagens, type IX and XI, are closely associated with CII fibrils and support the tensile strength of the CII network. Type VI collagen (CVI) also forms a fibrillar network, however it is preferentially located in the pericellular matrix of cartilage. Proteoglycans (PGs) constitute 10% of cartilage dry weight. Aggrecan, a chondroitin sulphate (CS)/keratin sulphate (KS) PG, is the major non-collagenous component of cartilage. It interacts with hyaluronic acid (HA) and link protein to form large aggregates within the collagen framework of the ECM. The negatively charged glycosaminoglycans (GAGs) of the aggregates create an osmotic swelling pressure which enables the tissue to become highly hydrated, giving cartilage its compressive resistance. Other PGs are expressed in cartilage, including versican, glycan, decorin and fibronectin (FN). These smaller PGs interact with other components of the ECM, for example collagen, and are involved in providing a structurally stable support for chondrocytes and modulating cellular behaviour by interacting with cell surface receptors (Heinegard 1989, Horton 1989).



Chondrocyte-ECM interactions are important in the regulation of cartilage function. Amongst the important molecular interactions are those involving the cell surface adhesion molecules CD44 and integrins.

In cell types other than chondrocytes, integrins have been shown to play a role in the regulation of many cell functions, including embryonic development, maintenance of tissue architecture, differentiation, proliferation, programmed cell death, tumour metastasis, and the response of cells to mechanical stress (Solursh 1989). The interaction of integrins with ECM molecules, including FN and collagen, results in the clustering of integrins and the formation of focal adhesion complexes, where integrins link the ECM and cytoskeleton. The assembly of focal contacts plays an important role in modulating cell adhesion and regulating changes in cell shape involved in cell spreading and locomotion. Focal contacts also serve as a framework for the association of signalling molecules involved in integrin regulation of cell behaviour. Protein phosphorylation is amongst one of the earliest events activated, involving molecules including focal adhesion kinase (FAK) and protein kinase C (PKC). Other events include changes in intracellular calcium concentration and pH, and alterations in gene expression (Clarke 1995).

In cartilage, integrin-ECM interactions are thought to be important in many aspects of chondrocyte function. Integrins mediate chondrocyte adhesion to many ECM proteins. As well as interacting with other components of the ECM, molecules which are localised specifically to the pericellular matrix, immediately adjacent to the chondrocyte, for example CVI (Poole 1988, 1992) and FN (Clemmensen 1982), interact with the chondrocyte via cell surface receptors, including  $\alpha 1$ ,  $\alpha 3$ ,  $\alpha 5$  and  $\beta 1$  integrins (Salter 1992, Loeser 1995, Enomoto 1993). *In vitro* chondrocyte spreading and migration on CII and FN substrates is mediated by interactions with  $\beta 1$  integrins (Enomoto 1997). Integrins are thought to be involved in the regulation of cartilage matrix synthesis and integrity, for example inhibition of integrin function also inhibits CII synthesis by chondrocytes in culture (Beekman 1997). However integrins are also involved in cartilage breakdown. FN fragments stimulate chondrolysis and decrease PG synthesis in cartilage explants, which is blocked by inhibitors of FN-integrin interaction (Homandberg 1994). Other chondrocyte

processes, for example proliferation and chondrogenesis, involve integrin interaction with the ECM. The interaction of  $\alpha 5 \beta 1$  integrin with CII and FN is essential for *in vitro* chondrocyte colony formation and DNA synthesis (Enomoto 1997). *In vitro* chondrogenesis is inhibited by function blocking anti- $\beta 1$  integrin antibodies (Shakibaei 1998), and expression of specific integrin subunits changes to correlate with the type of collagen synthesised (Shakibaei 1995). Chondrocyte  $\beta 1$  integrin-ECM interactions are required for chondrocyte survival, matrix deposition and differentiation in models of chondrocyte development (Hirsch 1997). Integrins have been shown to regulate chondrocyte production of cytokines *in vitro*, for example FN stimulated production of IL-6, which is mediated by interactions with  $\alpha 5 \beta 1$  integrins at the cell surface (Yonezawa 1996). Ligation of  $\alpha 5 \beta 1$  integrin with FN in chondrocyte cultures results in the formation of focal adhesion complexes comprising actin, FAK and the G protein Rho (Clancy 1997), although much less is known about the intracellular signalling pathways which follow. Signal transduction through integrins is known to upregulate metalloproteinase expression, which is mediated through IL-1 binding to its cell surface receptor (Arner 1995).

Similarly, CD44 is involved in several cellular functions, including adhesion, T cell activation, differentiation and embryogenesis. Its major ligand is HA, but it also binds to FN and CVI. Binding of HA results in the activation of several intracellular pathways, including serine phosphorylation of CD44, GTP binding, changes in calcium concentration, alterations in the cytoskeleton, and release of cytokines. The presence of many of CD44's ligands in the pericellular matrix of cartilage suggests that it may also act in the regulation of chondrocyte function in a similar manner (Lesley 1993). CD44 has been shown to play a role in chondrocyte adhesion, regulation of matrix synthesis and degradation, and cell proliferation. *In vitro* adhesion of chondrocytes to HA is mediated by CD44, which results in increased proliferation of cells and expression of proteins, including TGF- $\beta$  (Ishida 1997). In chondrocyte cultures IL-1, which inhibits PG synthesis, stimulates CD44 expression (Chow 1995). This suggests that stimulatory signals which regulate chondrocyte catabolism and matrix synthesis are mediated by CD44. In chondrocyte cultures and cartilage explants, inhibition of CD44 expression results in a decrease in

PG synthesis and matrix deposition, suggesting that CD44 is required for maintenance of matrix integrity (Chow 1998). Electron microscopy has also shown that antibodies which block CD44 function also inhibit chondrocyte pericellular matrix assembly (Knudson 1996).

Chondrocyte adhesion molecules therefore play many important roles in cartilage by mediating cell interaction with the ECM.

### **1.1.2. Osteoarthritis**

Osteoarthritis involves cartilage breakdown and progressive destruction of the joint. A number of factors, including trauma, metabolic abnormality, and genetic defects, can trigger the onset of osteoarthritis. In osteoarthritis, the protease content of cartilage increases, along with a downregulation in the levels of protease inhibitor. This leads to a net increase in protein degradation and fibrillation of cartilage. The tissue reacts to this destruction by remodelling. The chondrocytes assume a fetal phenotype; they increase in size and number, increase rates of DNA synthesis and proliferation, and form clones. (Poole 1993). The ECM is also altered. Cleavage of CII occurs. This is initially most pronounced in the superficial zone; however, with increasing severity of osteoarthritis the damage spreads to the mid and deep zones. The synthesis of new collagen increases to compensate for this loss, however the fibrils formed are thinner, appear disorganised and are eventually degraded. This results in a loss of tensile strength of the cartilage. Damage to the collagen network also leads to increased swelling of PGs and an increased tissue water content (Poole 1993).

PGs are also degraded, this loss occurring initially at the articular surface. As the disease progresses the PGs become larger and chemically different, often containing new structural epitopes on CS chains which are normally only found in fetal cartilage, suggesting that the degraded molecules are being replaced with newly synthesised PGs. In advanced osteoarthritis PG levels decrease further, with an eventual extensive reduction in PG content (Poole 1993).

The synthesis of other matrix components increases in an effort to repair tissue damage, for example FN (Burton-Wurster 1990), CVI (Swoboda 1998) and

tenascin (TN) (Salter 1993). The destruction of cartilage is accompanied by changes in growth factor and cytokine receptor expression, suggesting that these molecules are involved in the pathogenesis of the disease (Poole 1993).

The expression of cell adhesion molecules changes in OA cartilage. Immunohistochemical staining of articular cartilage has shown that CD44 expression in normal cartilage varies between the superficial and deep zones. An upregulation in the expression of CD44 in the chondrocytes located in the deep zones of OA cartilage results in a uniform expression throughout the whole tissue (Ostergaard 1997a). Integrin expression also changes in OA cartilage. Studies carried out *in vivo* and *in vitro* have shown that  $\beta 1$  integrin expression increases in OA cartilage (Loeser 1995) and  $\alpha 2$  and 4 and  $\beta 2$  integrins, which are not expressed in normal cartilage, are expressed in OA cartilage (Ostergaard 1998). This suggests that normal cell-ECM interactions are perturbed during the disease process, and this may play a role in the onset and progression of osteoarthritis.

The progression of osteoarthritis occurs as a result of the increased rate of synthesis of ECM being insufficient to compensate for the degradation, resulting in net loss of PGs and other proteins from cartilage, and the tissue is destroyed (Heinegard 1989). Changes in ECM content and expression of cell surface receptors affects cell-ECM interactions and the regulation of chondrocyte function by cell adhesion molecules, and therefore may be important in the development and progression of osteoarthritis.

### **1.1.3. NG2/Human melanoma proteoglycan (HMPG) and human cartilage**

Monoclonal antibody technology (Kohler 1975) has identified the antigen NG2/HMPG. NG2 is a unique single span transmembrane chondroitin sulphate rich proteoglycan, expressed by cells as both a 250 kD core protein and a chondroitin sulphate proteoglycan (CSPG) of molecular weight greater than 400 kD (Stallcup 1983). It shows restricted distribution, being seen in rat neuronal and glial cells and developing mesenchymal tissues, including cartilage (Stallcup 1983, Nishiyama 1991a). NG2 is however lost from cartilage during maturation, and is not expressed

by chondrocytes in adult rat cartilage (Nishiyama 1991a). Recent studies have shown NG2 to have significant homology with HMPG, a molecule expressed by melanoma cells but by few other adult human tissues (Nishiyama 1991b).

Evidence is increasing for roles for NG2/HMPG in cell matrix interactions. The observation that NG2/HMPG is present in developing rat cartilage, and has been shown to be a receptor for CVI, CII and FN molecules (Burg 1996, Iida 1995), recognised to be important in cartilage structure and regulation of chondrocyte function, raises the possibility that this molecule may have previously unrecognised roles in human articular cartilage. Furthermore the changes in expression of ECM molecules and cell adhesion molecules in OA cartilage, where chondrocytes are known to alter expression of cell adhesion molecules to that reminiscent of fetal cartilage, raises the possibility that NG2/HMPG expression may be seen during the onset and progression of osteoarthritis.

## 1.2. CELL AND TISSUE DISTRIBUTION OF NG2

### 1.2.1. Expression in the rat central nervous system (CNS)

Immunological techniques have been used to identify specific surface markers to classify cell types and trace cell lineages in the CNS. Nitrosoethylurea-induced cell lines derived from rat CNS (Schubert 1974) were screened using ion flux assays and divided into excitable (nerve) and non excitable (glial) types. Antisera were produced which recognised N or G antigens expressed by either neurones or glia respectively (Stallcup 1976). However some cell lines could not be defined by simple classification. These did not generate action potentials or express N antigens, which is indicative of a glial cell type, but did express voltage dependent sodium channels typical of neurones (Arner 1981). Antibodies were raised against three pseudo glial/neuronal cell lines B9, 49 and 92 (Wilson, S.S. 1981a, Stallcup 1983), one of these recognised an antigen NG2, so called as it is expressed only by cell lines with characteristics of both nerve and glial cells. Cell lines which exhibit properties of both nerve and glial cells are proposed to be undifferentiated precursors that can become either cell type. NG2 is therefore a potentially useful marker of CNS progenitor cells (Stallcup 1983).

*In vitro* cultures of embryonic and postnatal rat whole brain and isolated cerebrum and cerebellum contain NG2-expressing cells, which have properties of both glia and neurones (Stallcup 1981, 1983). This indicates that they may be developmental precursors similar to cell lines.

*In vivo* NG2 is expressed by normal rat brain (Wilson S.S 1981a). NG2 is first identified in the embryo. The number of NG2-positive cells increases during development, peaks at postnatal day 4/5 and decreases thereafter until postnatal day 19. Thereafter expression is down regulated by 50% to a steady state which is maintained in the adult (Levine 1987a). NG2-expressing cells have been reported to resemble astrocytes (Levine 1987a) and stellate and basket interneurones (Stallcup 1983).

Environmental conditions can influence the phenotype of cells which express NG2. Cerebellar cultures isolated from postnatal rats and grown in fetal calf serum



(FCS) become type 2 fibrous astrocytes, but without serum they become type 1 smooth protoplasmic astrocytes. This suggests that plastic cells that can acquire mature astrocyte properties express NG2 (Levine 1987b). This exogenous modulation of phenotype is similar to that of O-2A precursor cells identified in optic nerves which differentiate into either oligodendrocytes or astrocytes *in vivo* (Raff 1983, Stallcup 1987). O-2A cells isolated from rat postnatal optic nerves express NG2. When grown in FCS they mature into type 2 astrocytes and retain NG2 expression, but in serum free conditions they lose NG2 and cells mature into oligodendrocytes (Raff 1983, Stallcup 1987).

NG2-positive O-2A progenitor cells are detected *in vivo* in the rat cerebellum, from the last 3-4 days of gestation until the first 10 postnatal days (Stallcup 1987). Some blood vessels also express NG2 over this time (Miller 1995). The human cerebral cortex contains O-2A cells with similar features to NG2-positive rat cerebellar cells, suggesting that NG2 may also be expressed in the human CNS (Ong 1996).

NG2 has been used as a marker for O-2A precursor cells to monitor the immortalization of precursor cell lines (Redies 1991), and to identify O-2A cells in the study of cell-cell interactions in the development of the brain. Neuronal cells present in the cerebellum influence the differentiation of progenitor cells. A-neuronal optic nerve O-2A cells differentiate into oligodendrocytes in the absence of serum. Cerebellar O-2A cells do not differentiate into oligodendrocytes unless neurones are removed from the culture, illustrating the importance of cell-cell interactions in differentiation (Levine 1989).

### **1.2.2. Expression in the rat developing limb**

Immunohistochemical staining of a wide range of rat tissues showed that NG2 is expressed outside the CNS, predominantly in tissues of mesenchymal origin, including developing cartilage, skeletal muscle, smooth muscle and heart. NG2 expression in the developing rat limb was further studied. Apart from some weak reactivity in the endothelial and smooth muscle, all NG2 is found in the chondrogenic tissue. In the limb expression is developmentally controlled, with high levels of

protein and 8.9 kb mRNA expressed in the late embryonic and early postnatal rat by differentiating chondrocytes. As chondrocytes mature expression is down regulated (Nishiyama 1991a).

### **1.2.3. Expression in the rat vasculature**

NG2 is expressed by rat embryonic blood vessel cells *in vivo*. In brain capillaries the endothelial cells are NG2-positive, up to the second postnatal week. In contrast, in the capillaries of the spinal cord and aorta of embryonic, postnatal and adult rats, NG2 is found in the smooth muscle cells adjacent to the endothelial cells. *In vitro* cultures of smooth muscle cells from postnatal rat aorta express NG2. This suggests a difference in cell type and timing of NG2 expression within and outside the CNS (Grako 1995).

### **1.2.4. Expression in rat chondrosarcomas**

Rat chondrosarcoma cells express NG2 but normal adult rat chondrocytes do not (Leger 1991).



## **1.3. PHYSICAL CHARACTERISTICS OF NG2**

### **1.3.1. Biochemical analysis**

NG2 can be precipitated as a well defined 300 kD band and a smeared 400-500 kD band from surface labelled NG2-expressing rat cell lines but not NG2-negative cell lines (Stallcup 1983), from optic nerve O-2A precursor cells (Stallcup 1987), from chondrosarcomas (Leger 1994, 1995) and from cultures of rat developing limb chondrocytes (Nishiyama 1991a). The high molecular weight component (HMWC) of NG2 is a CSPG, as indicated by labelling with radioactive sulphate and the accumulation of the 300 kD protein in the presence of agents which prevent GAG attachment to core protein (p-nitrophenylxyloside) or remove CS GAGs (chondroitinase ABC (cABC)). Both components of NG2 are glycoproteins, as they are labelled with radioactive galactose and mannose. NG2 can be extracted from cells using non-ionic detergent but not ethylene diaminetetraacetic acid (EDTA), suggesting that it is tightly associated with the membrane. A smaller form of 290 kD is secreted and can be detected in the tissue culture medium (Stallcup 1983).

### **1.3.2. Biosynthesis**

Pulse labelling of B49 rat cell lines with radioactive leucine shows that a 260 kD protein is made. This is N-glycosylated with immature unbranched high mannose oligosaccharides to form a 275 kD, tunicamycin- and endoglycosidase H (Endo H)-sensitive glycoprotein. The addition of sialic acid forms the 300 kD, neuraminidase sensitive, Endo H resistant glycoprotein, after which the addition of GAGs yields the HMWC. The HMWC is detected after 15 min and increases in intensity upto 30 min at which point the 275 kD glycoprotein ceases to be detected (Stallcup 1983).

### **1.3.3. Cell surface localisation**

NG2 is distributed in a speckled pattern over most of the surface of B49 cells (Stallcup 1983) and in embryonic and postnatal rat brain primary cultures (Stallcup 1981). It is expressed at the cell surface of rat cerebellum *in vivo* (Levine 1987a).

## **1.4. CELL AND TISSUE DISTRIBUTION OF HUMAN MELANOMA PROTEOGLYCAN (HMPG)**

### **1.4.1. Expression in human melanoma cells**

Malignant transformation results in the expression of novel antigens by cells. In particular PG expression varies between normal and transformed cells (Natali 1981). Many polyclonal and monoclonal antibodies have been raised to melanoma specific antigens, which have identified the presence of a unique PG in human melanoma cells (TABLE 1.1.).

These melanoma-specific antibodies recognise the same antigen, HMPG (Harper 1982, 1984, Herlyn 1987, Imai 1980, 1981, Johnson 1982, Morgan 1986, Nagelhus 1993, Ng 1982, Real 1985, Ross 1982, 1984), which is similar to other melanoma-associated antigens described (Bumol 1982, Carrel 1980, Hellstrom 1983, Ng 1982). Much of this information was consolidated at an anti-melanoma workshop using pooled resources, and excellent agreement between labs was achieved. Epitope analysis revealed that antibody 9.2.27 recognises a different epitope than 225.28S, 763.24TS (Harper 1982) and 155.8 antibodies (Harper 1984). The epitope recognised by 9.2.27 is more widely distributed than those recognised by other monoclonal antibodies (Harper 1982, 1984, Kantor 1982). The expression of HMPG was studied using these melanoma-specific antibodies, and found to be of limited distribution in human cells (Carrel 1980, Giacomini 1984, Harper 1984, Hellstrom 1983, Herlyn 1983, Imai 1981, Koprowski 1978, Morgan 1981, Natali 1981, 1983, 1984, Real 1985, Ross 1984, Wilson B.S 1981b). (TABLES 1.2. and 1.3.). Extensive screening of fresh tissue and cultured cells indicates that HMPG has a unique distribution, it is most strongly associated with melanoma cell surfaces from which it is also secreted, both *in vitro* and *in vivo*. All reactivity to the antibodies was specific for melanoma cells with the exception of some weak blood vessel staining. HMPG is not expressed by normal adult or fetal melanocytes, unless the cells are stimulated to proliferate with cholera toxin (Lloyd 1982), tumour inducer 12-O-tetradecanoylphorbol-13-acetate (Real 1985) or other phorbol esters (Kantor 1982). HMPG is not expressed by any other normal or tumour cell types tested.

**TABLE 1.1.**

Monoclonal and polyclonal antibodies raised against human melanoma cells, which recognise the antigen HMPG.

ANTIBODY	IMMUNOGEN	REF.
Polyclonal antiserum 6522	M21 cell lines	McCabe 1978, McCabe 1979
9.2.27	M14 melanoma cell line extracts with competing antigens, FN and HLA removed.	Morgan 1981
155.8	M14 cell line membranes	Harper 1984
48.7	M1733 melanoma cell lines	Hellstrom 1983
225.28S, 138.13, 149.53	M21 melanoma cell lines	Wilson 1981
653.40S, 165.28T, 653.25N	M21 melanoma cell lines	Imai 1981
O1-95-45, 691-19-19	Melanoma-mice cell hybrids	Koprowski 1978
M28B5	SKMEL 93 cell lines	Houghton 1982
AMF-6, NKI.M6	melanoma cell lines	DeVries 1986
a-mel/4, a-mel/5, Mel-14	Me-43 melanoma cell lines	Carrel 1980
15-43, 18-90, 95-45, 19-19, 60-23	melanoma cell lines	Herlyn 1983
PG-2, 3, 4, ZMEO18	melanoma cell lines	Morgan 1986
473.54S, 653.40S, 730.25T, M23/353, M19/152.6, 763.74S	M21 and Colo 38 melanoma cell lines	Kantor 1982
763.24TS	melanoma cell lines	Harper 1982
Mel-14	melanoma cell lines	Herlyn 1982
G7A5	melanoma cell lines	Johnson 1988
ME31.1, ME126	WM46 melanoma cells	Ross 1984, Herlyn 1988

**TABLE 1.2.**

Expression of HMPG *in vitro*. HMPG was identified by immunological staining of cells in culture and shows a restricted distribution, being expressed predominantly by melanoma cell lines.

<b>HMPG +VE CELLS</b>
metastatic and primary melanoma cells lines (A375, BE, BP, BwV, CAR#2, Colo 38, Colo 55, IGR3, LIN, M10, M14, M16, M21, M51, Me-8, Me-33, Me-43, Me25-1, Me-85, Mel-2AM, Mel-2AP, Mel-Ei-78, Mel-57, Mel-67, MP-6, MP-8, SCRF, Skmel-1, Skmel-23, Skmel-25, Skmel-93, WM9, WM22, WM28, WM35, WM75, WM115, WM164, WM165, WM272) capillary endothelium (weak) melanoma cell culture medium neuroblastomas
<b>NORMAL ADULT HMPG -VE CELLS</b>
B cell lines, kidney fibroblasts and epithelium, melanocytes, T cell lines, skin fibroblasts
<b>NORMAL FETAL HMPG -VE CELLS</b>
brain, erythroblastoid cell lines, fibroblasts, melanocytes, human lymphoid, B lymphoblasts, fibroblasts, erythrocyte cell lines.
<b>TRANSFORMED HMPG -VE CELLS</b>
Astrocytoma carcinomas of B cell, bladder, breast, cervical, chorio, colorectal, endometrial, epithelial cells, fibroblastoid, GI, heart, HELA, lung, mammary, ovarian, pancreatic, prostate, renal, T cell, thyroid, vulva, chemoectoma, EBV transformed malignant lymphocytes and erythrocytes, glioblastoma, hypernephroma, retinoblastoma sarcoma fibroblasts

**TABLE 1.3.**

Expression of HMPG *in vivo*. HMPG was identified by immunological staining of a panel of frozen sections taken from a wide range of tissues. Expression is limited to melanoma tumour cells and blood vessels.

<b>ADULT HMPG +VE TISSUES</b>
astrocytoma, blood vessels, primary, metastatic and recurrent melanoma (cutaneous, mucosal and uveal) tumour cells, serum (from melanoma patients, normal patients and non melanoma patients), senile freckle, skin carcinomas
<b>NORMAL ADULT HMPG -VE TISSUES</b>
bladder, breast, cartilage, duodenum, endometrium, epidermis, lymph node, lymphocytes, melanocytes, ovary, peripheral nerve, salivary gland, normal skin, stomach, testes, tonsil
<b>NORMAL FETAL AND ADULT HMPG -VE TISSUES</b>
adrenal gland, brain cortex, colon, pancreas, kidney, lung, nerves, squamous cells or dermal collagen, heart, kidney, liver, lung, lymph node, oesophagus, pancreas, prostate, skeletal muscle, skin, smooth muscle, spleen, thyroid, uterus
<b>TRANSFORMED HMPG -VE TISSUES</b>
<i>benign</i> : breast dysplasia and cyst, fibroma of soft tissue, interstitial pneumonitis, intestinal polyp, lymphangioma, neurinoma, prostatic and thyroid adenoma, skin lesions (dysplasia, basal cell papilloma keratoacanthoma, verruca vulgaris, leukoplakia)
<i>malignant</i> : bladder, breast, endometrial, epithelial, gastro-intestinal, kidney, liver, lung, ovary, squamous cell and testes carcinoma, meningioma, neuroblastoma, astrocytoma, glioblastoma, leukaemia, liposarcoma, rhabdomyosarcoma, sarcoma
neoplastic tumour types of non melanocyte origin (sclerosing angiomas, warts, basal cell and squamous carcinoma)

Although the antibodies against HMPG were derived using metastatic cutaneous melanoma, HMPG is expressed by all types of melanoma tumour tested, including mucosal melanoma from the head and neck (Henzen-Logmans 1988), uveal melanoma (Natali 1989), acral lentiginous melanoma, and nodular melanoma lesions (Kageshita 1991).

HMPG is expressed by metastatic, recurrent and primary melanomas. Expression has been reported to remain constant in tumours of all sizes (Johnson 1988), in lesions from different sites in the same patient, and to have no correlation with degree of transformation or prognosis (Giacomini 1984, Morgan 1986, Natali 1983, 1985, Wilson 1982, Ziai 1987). However some evidence suggests that expression is increased in metastatic melanoma compared to benign lesions (Kageshita 1991, Wilson 1982), with significantly shorter patient survival in HMPG positive cases (Kageshita 1993, 1994).

HMPG is secreted by melanoma cells lines (Giacomini 1983, Imai 1981, Natali 1981, Wilson 1983) and is also found in the serum (Giacomini 1984). The highest levels of HMPG in the serum are detected in patients with advanced metastatic melanoma (Herlyn 1982) or gross tumour bearing patients, when compared to normal donors, or patients with other cancers (breast, lung, cervix and prostate carcinoma) or with non malignant disease (Ross 1984). Secretion of HMPG was increased by human leukocyte interferon (Matsui 1987). The significance of the shedding of HMPG into serum may be significant in affecting distant cells, but its function in the circulation is unclear.

Expression of HMPG was maximal during G2 and M cell cycle phase of melanoma cell lines Colo 38, M16 and M21 (Burchiel 1982). Higher amounts of HMPG were seen in cells that had the medium changed more frequently, where no contact inhibition occurred and which were significantly larger, suggesting a decrease in less healthy cells (Lindmo 1984). Hyperthermia (42°C) reduces HMPG expression up to 24 h after treatment at which point it returns to normal levels (Davies 1985). Ligation of HMPG by 9.2.27 induces internalisation of HMPG at 37°C. This is increased by hyperthermia (Davies 1990). Hypoxic conditions and photoactivated hematoporphyrin derivatives reduce HMPG expression (Davies 1988).

HMPG shows limited expression in the human tissues studied. However it has been identified in a few cell types other than melanoma by the antibodies M28B5, 9.2.27, 225.28S, Mel 14, O-95-45, AO92 and 122 (Garin-Chesa 1989) (**TABLE 1.4.**)

#### **1.4.2. Expression in human pericytes**

HMPG expression in blood vessels in the tumour stroma (Real 1985) is restricted to the pericytes of capillaries and venules (Schlingemann 1990), which are similar to smooth muscle cells of arteries (Sims 1986). Pericytes of other lesion types associated with vascular proliferation or angiogenesis, for example granulation tissue of wound healing and synovitis, also acquire HMPG, but normal capillaries of the kidney, muscle, thyroid, placenta, and colon or ovarian carcinoma do not (Schlingemann 1990).

#### **1.4.3. Expression in Acute Myeloid Leukaemia (AML) and Acute Lymphoblastic Leukaemia (ALL) blasts**

Malignant AML blasts express different antigens from normal mature cells. They are not novel proteins but are expressed during blast development. HMPG is specifically expressed by transformed marrow cell lines and some cell lines of non hematopoietic origin, such as those derived from smooth muscle, marrow stroma, cervical carcinoma and epithelial cells. It is not expressed by mature cells from normal adult marrow or developing cell lines of hematopoietic origin. This novel expression of HMPG by AML blasts but not normal haematopoietic cells at any point during development could prove to be significant in diagnosis and prognosis of AML cases (Smith F.O. 1996a).

Leukaemic marrow from children with AML that express HMPG all have the same 11q23 chromosomal abnormalities, namely myeloid/lymphoid leukaemia (MLL) gene rearrangement. The MLL gene is thought to encode a transcription factor that could control HMPG gene expression, alterations in which may lead to aberrant expression of HMPG. The prognosis of HMPG positive patients is poor, with a greater rate of death than HMPG negative patients (Hilden 1997).



ALL blasts from children with poor outcome often have MLL gene rearrangements (Borowitz 1993, Pui 1991), and also express HMPG. Blasts that do not have these specific alterations fail to express HMPG, as does normal lymph node tissue (Behm 1996). This unique reactivity with neoplastic leukocytes with MLL disruption could be clinically useful, as a highly specific and sensitive marker.

#### **1.4.4. Expression in human astrocytomas**

Astrocytoma cells express HMPG *in vivo* and *in vitro*, as detected by the monoclonal antibody AO122. This antibody also reacts with fibroblasts and adult and fetal brain homogenates. This antibody does not react with neuroblastomas, epithelial cancer cells of lung, breast, colon, renal, bladder and cervix origin, transformed B cells, T cell leukaemia, kidney epithelial cells or erythrocytes (Cairncross 1982).

#### **1.4.5. Expression in human glioblastoma and capillaries**

Adult human glioblastoma tissues express HMPG *in vivo*, in the proliferative endothelial cells, perivascular ECM and weakly in the glioma cells themselves. In contrast, glia, neurones, endothelial cells, endothelial basement membrane or structures in the peri-endothelial space of capillaries in normal brain do not express HMPG. HMPG is absent from other tumour types including low grade brain tumours and non proliferative tumours, for example anaplastic astrocytoma, medullablastoma, primary CNS lymphoma and meningioma, which lack endothelial proliferation. This suggests that HMPG is a marker of proliferative brain capillary endothelial cells in the most malignant transformed astroglial cells (Schrappe 1991).

#### **1.4.6. Expression in human sarcomas**

Anti-HMPG antibody 9.2.27 was included as a negative control in human sarcoma detection. However it showed significant binding to several human sarcoma lines and fibroblasts. Tissue sections from sarcomas, osteosarcomas, malignant schwannomas, malignant fibrous histiocytomas and synovial sarcomas also express HMPG, whereas normal fibrous connective tissues do not (Godal 1986).



**TABLE 1.4.**

Human tissues which show positive reactivity with anti-HMPG antibody M28B5. Expression of HMPG detected by M28B5 staining of frozen sections from normal and transformed human tissues. Expression is less restricted than previously thought, with a wide variety of cells showing strong immunoreactivity with M28B5 (Garin-Chesa 1989).

<b>NORMAL TISSUES</b>	<b>TUMOUR TISSUES</b>
Adrenal Cortex	Adrenal cortical carcinoma
Bile duct epithelium	Astrocytoma
Bronchial epithelium	Basal cell carcinoma
Capillary endothelium	Bladder carcinoma
CNS neurones, astrocytes	Breast carcinoma
Fibroblasts (subset)	Chondrosarcoma
Intestinal mucosa	Colon carcinoma
Keratinocytes	Embryonal rhabdomyosarcoma
Kidney tubules	Gastric carcinoma
Leydig cells	Leiomyosarcoma
Mammary glands acini, ducts	Lung carcinoma
Myoepithelial cells	Melanoma
Pancreatic acini	Meningioma
Parotid gland ducts acini	Mesothelioma
Pneumocytes	Nevus
Schwann cells	Osteogenic sarcoma
Seminiferous tubules (weak)	Ovarian carcinoma
Skeletal muscle	Prostate carcinoma
Skin appendages	Rhabdomyosarcoma
Smooth muscle	Squamous cell carcinoma
Thyroid epithelium	Synovial sarcoma
	Thyroid carcinoma

## 1.5. PHYSICAL CHARACTERISTICS OF HMPG

### 1.5.1. Biochemical analysis

HMPG comprises two components which are expressed simultaneously. A 250-280 kD protein and a heterogeneous HMWC (>400 kD) can be immunoprecipitated from the surface of melanoma cell lines, (Bumol 1982, Harper 1984, Hellstrom 1983, Kantor 1986, Koprowski 1978, Morgan 1981, 1986, Natali 1981, Ng 1982, Real 1985, Ross 1982, 1984, DeVries 1986, Wilson 1981, 1982, Ziai 1987), cultured pericytes from human adult brain (Schlingemann 1990), AML and ALL blasts (Smith F.O 1996a), glioblastoma cell lines and brain capillary endothelial cells from glioblastoma tissue (Schrappe 1991), and cultured sarcoma cells (Godal 1986). A 210 kD protein is precipitated from cultured fibroblasts, indicating a less modified form is expressed by these cells (Godal 1986).

HMPG is also secreted from melanoma cells. Both the HMWC (Hellstrom 1983) and the 280 kD protein (Ng 1982, Ross 1984, Wilson 1981, Ziai 1987) have been detected in the tissue culture medium of melanoma cells, which is increased by interferon (Wilson 1983). HMPG has a dual distribution, being expressed both at the cell surface and released from cells *in vivo* and *in vitro*.

The HMWC is a CSPG, first suggested by its large size and confirmed by sulphate labelling of melanoma cells, susceptibility of O-linked GAG groups to  $\beta$  elimination reactions, and loss of the HMWC after digestion with cABC or treatment of the cells with monensin (a monovalent ionophore which inhibits cell surface expression of PGs) (Bumol 1982, Harper 1984). GAG analysis by cellulose acetate electrophoresis against heparin sulphate (HS), CS and HA standards also confirmed that HMPG is a CSPG (Harper 1984). High performance liquid chromatography (HPLC) against co-migrating standards revealed that HMPG contains both delta Di-6S and -4S sulphate types (Bumol 1982). The HMWC barely entered a 5% acrylamide gel, and 3% acrylamide and 0.75% agarose gels show that HMWC consists not of a single component but of a disperse smear ranging from 300-700 kD (Wilson 1983), implying that 3-12 60 kD GAG chains can be attached to the protein (Garrigues 1986, Ross 1983).

The 250 kD protein is not sulphated but is susceptible to neuraminidase indicating that it is an N-linked, sialated glycoprotein (Bumol 1982). This protein has a isoelectric point of 6.9, is a polar molecule with a high leucine, low lysine content and is heat and trypsin sensitive (Ross 1983).

Treatment with cABC results in the loss of the HMWC and a qualitative increase in the 250 kD protein, suggesting that the HMWC has been converted into the 250 kD protein. A similar result occurs upon addition of p-nitrophenylxyloside, an exogenous GAG chain acceptor. Tryptic peptide map analysis of digests of both the 250 kD protein and CSPG confirm that they consist of identical peptides. HMPG is therefore expressed as two forms at the cell surface, a CS free glycoprotein core which, upon GAG addition, forms the CSPG (Ross 1983).

### **1.5.2. Cell surface localisation**

HMPG is distributed in a punctate pericellular pattern on melanoma cell surfaces (Bumol 1984). At higher magnification HMPG is seen in filamentous structures that interact with the underlying substratum (Harper 1984), suggesting a functional role at discrete areas of the cell surface. Reactivity with anti-HMPG antibody was seen in all cells but was heterogeneous, with greater intensity in some cells (Harper 1984). Ultrastructural study of the distribution of both HMWC and unmodified core in melanoma cells revealed an expression restricted to clusters on upper, peripheral and lower cell surface projections, but no expression on smooth membrane areas or blebs (Garrigues 1986, Hellstrom 1983). No co-localisation of HMPG with FN could be detected (Bumol 1984). Focal adhesion plaques do not contain HMPG (Cheresh 1984). Light and electron microscopy revealed that HMPG distribution is limited to microspikes in clusters on the cell membrane of cultured pericytes (Schlingemann 1990). Expression in glioblastoma cells is localised to structures on the outer surface of the basement membrane and the subendothelial space of the small capillaries, suggesting a functional role in cell-ECM interactions. HMPG expression *in vitro* on glioblastoma cell lines is located in a punctate pericellular distribution, and quantification of 9.2.27 binding revealed 10,000-400,000 binding sites per cell

depending on the cell line type, compared to 600,000 for M21 melanoma cells (Schrappe 1991).

### **1.5.3. Biosynthesis**

The biosynthesis of HMPG was studied by pulse chase analysis using antibodies 9.9.27, 149.53, 225.28S and 763.74T which recognise intracellular precursors of HMPG and fully processed products (Bumol 1982,1984, Kantor 1986). In the first 10 min after pulse, 210, 220, 240 kD Endo H sensitive proteins are synthesised with high mannose, non processed oligosaccharides attached. At 20 min a Endo H resistant 250 kD protein and the HMWC are detected, which possess processed complex oligosaccharides. At 60 min the levels of the 250 kD protein remain constant but production of the HMWC increases, up to 120 min when synthesis levels off. At 48 h both the 250 kD protein and the HMWC are present, however the 250 kD protein is lost at 72 h, when only the HMWC can be detected. No proteins are detected from 72 h up to 96 or 148 h. This half life of expression at the cell surface is unusual compared to typical PG half lives of 70-90 min (Bumol 1984).

Monensin treatment of melanoma cells causes inhibition of mature CSPG assembly and 250 kD protein maturation, and results in an arrest in HMPG synthesis with a 245 kD Endo H resistant precursor (Bumol 1984, Harper 1986). Ammonium chloride disrupts ATP-driven membrane-bound proton pumps that acidify intracellular vesicles. Treatment of melanoma cells with ammonium chloride results in normal HMPG synthesis up to expression of the 250 kD protein at the cell membrane, but no CSPG is made. (Harper 1986). This shift towards the CS GAG free form suggests that ammonium chloride acts after the maturation of the core protein, but before the addition of GAGs which occurs in a low pH dependent compartment of the golgi. GAG addition is not a prerequisite for HMPG cell surface expression, nor a sorting signal, leading to speculation about the function of the GAG chain, and which form of the PG is functionally active. Diethylcarbamazine (DEC) inhibits the secretion of PGs from cells and the addition of GAGs in melanoma cells (Spiro 1986); HMPG synthesis is halted at the Endo H sensitive 240 kD step, no 250 kD protein or CSPG is made. DEC acts at a point where N-linked oligosaccharides

are processed, perhaps by inhibiting transport from the endoplasmic reticulum to the golgi (Spiro 1986). Tunicamycin, which inhibits N-linked glycosylation, results in the expression of an Endo H resistant 200 kD protein, suggesting that the unmodified core is not competent for GAG chain addition (Spiro 1989). Cells treated with glucosidase I and II inhibitors accumulate a 240 kD Endo H sensitive, high mannose form of HMPG, suggesting that removal of glucose is required to allow GAG addition. In contrast mannosidase inhibitors show no effect on the ability of the cell to produce CSPG, suggesting that proteins which have not been fully processed to the complex form still acquire GAGs. Transport to the cell surface is inhibited by glucosidase but not mannosidase inhibitors, suggesting that initial trimming but not final processing is required. This indicates that glucose removal is an important step in signalling of intracellular transport of the core to the site of GAG addition and subsequently to the cell surface (Spiro 1989).

Combinations of inhibitors have mapped the synthesis of HMPG as well as deriving useful information about PG synthesis in general. HMPG synthesis occurs via several metabolic intermediates and vesicular shuttling pathways between intracellular compartments which house modifying and processing enzymes to the cell surface.

## 1.6. COMPARATIVE ANALYSIS OF NG2/HMPG

The complete cDNA sequence of NG2 was determined, from which the primary structure was deduced (Nishiyama 1991b). A short sequence of the amino terminal of HMPG was reported to be identical to that of NG2 (Nishiyama 1991b), and when the primary structures of the two proteins were published they were found to be identical.

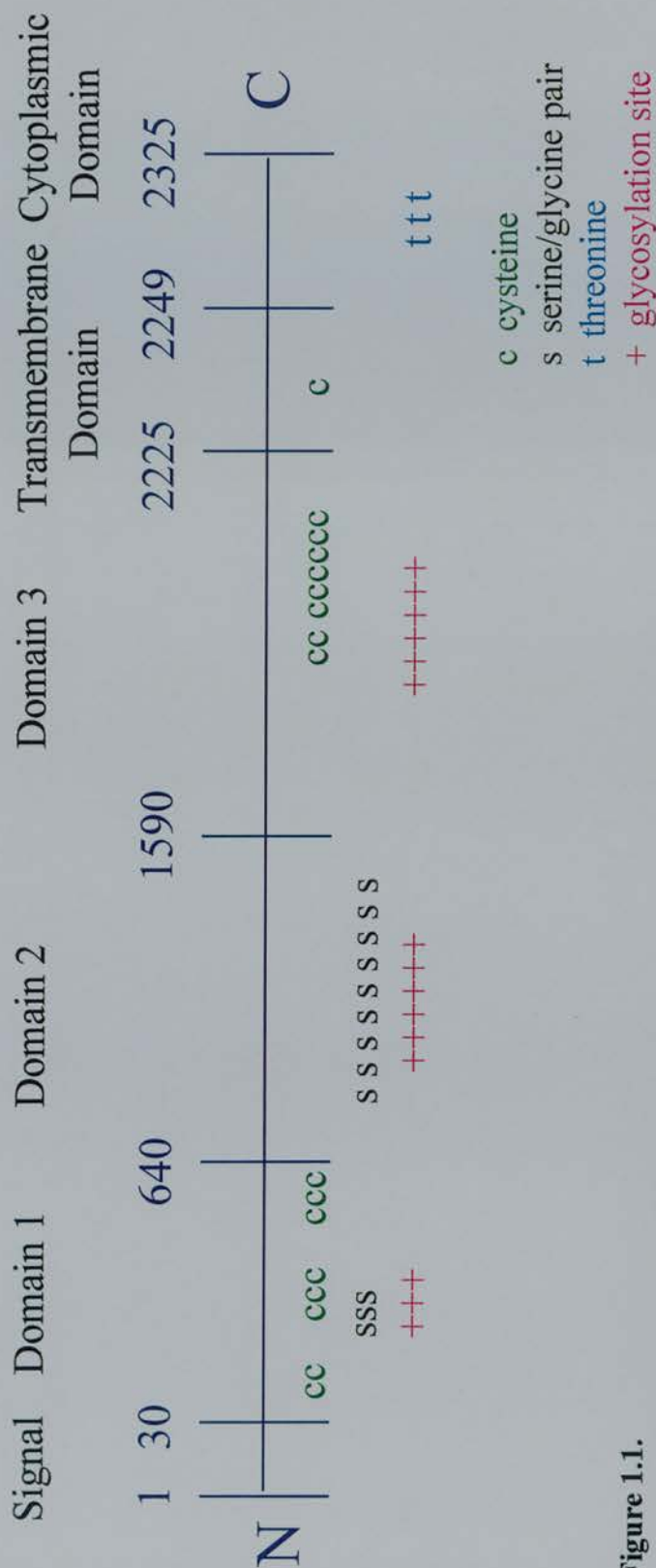
### 1.6.1. Primary structure of NG2

Antibodies raised to intact NG2 and fragments of NG2 purified from B49 rat cell lines were used to immunoscreen B49 cDNA libraries. An NG2-expressing clone was used to isolate the entire sequence of NG2. It is expressed as a 8.9 kb mRNA in NG2-positive cell lines and consists of 8071 nucleotides and 2325 amino acids (AA) giving a calculated MW of 251,470 D. (**FIGURE 1.1.**)

#### *Transmembrane Domain.*

Hydropathy analysis reveals a putative signal peptide following the initiation codon (ATG) at the N terminus and a second hydrophobic sequence near the C terminus. The latter sequence comprises 25 hydrophobic AA followed by several basic AA which meet the criteria for a transmembrane domain. Within the transmembrane domain there is a single cysteine, which is also found in CD44 (Goldstein 1989), syndecan (Saunders 1989) and the FGF-binding protein (Kiefer 1990), the function of which is unknown. Antibodies against N and C terminal peptides were used to stain both live and permeabilised B49 cells to reveal that the N terminus is extracellular and the C terminus is intracellular.





**Figure 1.1.**  
**Primary structure of NG2/HMPG.**  
 NG2/HMPG is a 2325 amino acid protein which consists of a small cytoplasmic domain, containing 3 threonine residues (t) which are potential PKC phosphorylation sites. It has a single transmembrane domain, a large extracellular region and a signal sequence for sorting to the cell membrane. The extracellular segment is divided into 3 domains, all of which contain potential glycosylation sites (+). Two of the extracellular domains are rich in cysteine residues (c) and these are separated by a serine/glycine rich domain (s), which is the proposed site of GAG attachment.

### *Cytoplasmic Domain.*

The small cytoplasmic domain of NG2 consists of 76AA. It contains three threonine residues which together with surrounding AA conform to the motif for PKC phosphorylation sites. Threonine 2255 and its neighbouring AA most closely match the consensus sequence of R(K)-XXT(S)XR(K) (Kemp 1990). Database searches revealed no other homology between the cytoplasmic domain and any other protein.

### *Extracellular Domains.*

The larger extracellular region contains 15 potential N-linked glycosylation sites (NXS/T) and can be divided into 3 domains, two cysteine-rich domains separated by a serine/glycine-rich domain.

The first domain (AA 30-640) contains 8 cysteines with surrounding AA unlike those common in many large ECM or cell surface molecules, for example epidermal growth factor like repeats (Doolittle 1984), immunoglobulin like repeats (Williams 1988) or link protein sequences (Neame 1986). Other notable features include a short stretch of acidic AA between the 4th and 5th cysteines, which are considered unusual in extracellular regions. Their function is unclear, but similar sequences exist in the link protein-like globular domain, the GAG attachment site of versican (Zimmerman 1989) and basic fibroblast growth factor receptor (Lee 1989). There is also a hydrophobic cluster at AA 424-440. The first extracellular domain contains 3 of the 15 potential glycosylation sites.

The second domain (AA 641-1590) contains no cysteines but has 9 serine/glycine pairs. The AAs around serines 998 and 1342 conform to consensus sequences for CS GAG attachment sites (E/DGSGE/D) (Zimmerman 1989), or (SGXG) (Bourdon 1987). The second extracellular domain contains 6 of the 15 potential glycosylation sites.

The last domain (AA 1590-2325) contains 8 cysteines, 6 of which are clustered into a 35 AA stretch near the transmembrane domain. Cysteines that lie just outside the transmembrane domain have been implicated in the formation of intermolecular disulphide bonds, for example in the NOTCH protein of *Drosophila* (Kidd 1989), or intersubunit disulphide bonds, for example in insulin receptor-like

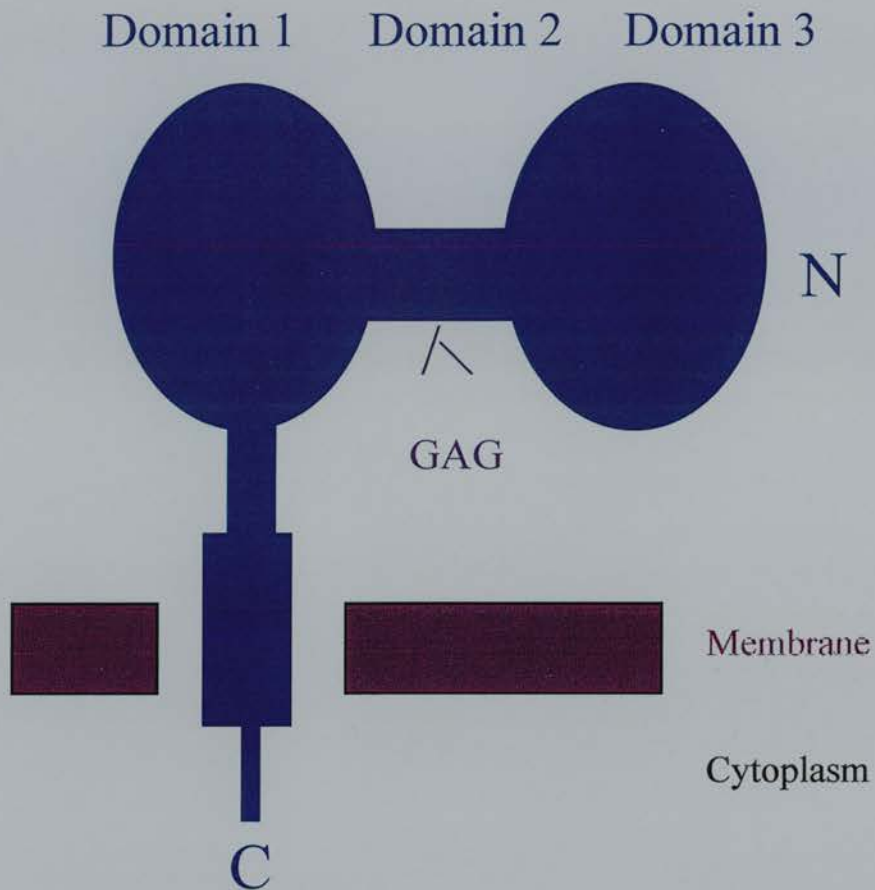


receptor (Shier 1989). Whether they perform these functions in NG2 is unknown. Data base searches revealed that AA 2050-2064 have 40% identity to a segment near the C terminal of the  $\alpha$  subunit of the human insulin receptor-related receptor (Shier 1989). In particular 3 cysteines are conserved in both these proteins and insulin receptor and insulin like growth factor I receptor. The third domain contains 1 potential GAG attachment site and 6 of the 15 potential glycosylation sites.

#### *Biochemical Analysis.*

A 300 kD protein can be precipitated from whole cell lysates of B49 rat cell lines. Mild trypsinisation of the cells releases a 120 kD N terminal cysteine-rich fragment. On reducing gels this fragment exhibits a slower motility indicating the presence of disulphide bond formation and a globular conformation in the native protein. This fragment is not affected by cABC treatment. Treatment of cells with EDTA releases a 200 kD fragment containing the N terminus and the serine rich region. In the absence of cABC this fragment forms a heterodisperse component of >400 kD, suggesting that it contains GAG attachment sites. Due to the lack of cysteines, this domain is likely to have an extended conformation similar to that predicated for the serine/glycine rich domains of other PGs (Doege 1987, Zimmerman 1989). This conformation was confirmed by electron microscope studies (Tillet 1997) (**FIGURE 1.2.**)

Southern blot analysis of genomic DNA isolated from rat cell lines revealed a single band indicating that NG2 is coded for by a single gene.



**Figure 1.2.**

**Structure of NG2/HMPG.**

NG2/HMPG is a single span transmembrane protein with a short cytoplasmic domain and a large extracellular region. The extracellular segment consists of 2 cysteine-rich domains which form globular configurations, separated by a rod like cysteine-free domain, which is predicted to form GAG binding sites.

### *Internal Repeats.*

A search for internal repeats in the sequence of NG2 revealed that there are four 200AA long negatively charged and glycine containing sequences that evenly span the extracellular region. These are similar to chick N-cadherin calcium binding domains. The region of highest homology was found in a 12 AA stretch at the C terminus of the second N-cadherin repeat. This has been proposed to form a  $\beta$  sheet followed by a  $\beta$  turn or coil, and be involved in calcium binding (Hatta 1988), suggesting a similar function in NG2. There is a 30 AA long repeat in the C terminal third of the extracellular domain which is unique to NG2. A tripeptide, FRV, appears five times in the extracellular domain of NG2 within the serine/glycine rich domain, four of which are clustered in the N terminal half of the area.

NG2 is a novel integral membrane protein with a large extracellular region and a small cytoplasmic domain. Unlike other large PGs, for example the syndecan family which have a common cytoplasmic domain (Kiefer 1990), NG2 shares little homology with any other protein, including other membrane spanning PGs (Fransson 1987) such as CD44 (Goldstein 1989), thrombomodulin (Jackman 1986) and the invariant chain of the class II histocompatibility antigen (Strubin 1984).

### 1.6.2. Primary structure of HMPG

HMPG was cloned and sequenced by screening cDNA libraries from M21 melanoma cells with a 4 kb rat NG2 cDNA probe. The resulting cDNA from a positive clone was used as a PCR primer to generate a series of overlapping clones, which DNA was sequenced and from which the 2322 amino acid sequence was deduced.

Northern blots of RNA from various sources were probed with HMPG cDNA. A 9 kb transcript is expressed only in HMPG-positive cell lines. No transcript was seen in any other cell type tested. *In situ* hybridisation of human melanoma cells using the same probe gave strong signals in all cell lines that reacted with anti-HMPG antibody.

HMPG is almost identical to rat NG2, with small variations found in the third domain. NG2 has a cluster of 6 cysteines at AA 2043-2091 which HMPG does not. In HMPG there are an additional 3 bases, the first at position 6128 causing a shift in the reading frame, which continues after the second at 6244, but is resolved after the third at position 6273 (Pluschke 1996).

## **1.7. FUNCTION OF NG2/HMPG -INTERACTION WITH ECM**

### **1.7.1. Cell surface localisation**

NG2/HMPG is localised to the cell surface of rat cell lines (Levine 1987a). In human melanoma cells NG2/HMPG is expressed at the cell surface in filaments/microspikes often connected to the underlying substratum (Harper 1984, Garrigues 1986). Microspikes make initial contact with adjacent cells or the underlying substratum, suggesting a role for NG2/HMPG in cell surface interactions (Albrecht-Buehler 1976). In non-transformed cells microspikes also mediate contact inhibition growth, but not in tumour cells (Albrecht-Buehler 1976). The presence of NG2/HMPG in tumour microspikes may account for the lack of contact inhibition.

### **1.7.2. Direct interaction of NG2/HMPG with ECM proteins**

Purified NG2/HMPG from melanoma cells interacts with HA (Bumol 1982). NG2/HMPG purified from B49 rat cell lines binds to CII, type V collagen and CVI, TN and laminin (LN) (Burg 1996). This is mediated by the protein core (Burg 1996). Decorin inhibits NG2/HMPG binding to these collagens but not to TN, suggesting that interaction between NG2/HMPG and collagen is mediated by a domain shared with decorin, but interaction with TN is at a site distinct from its collagen-binding domain. The only homology between NG2/HMPG and decorin is a leucine-rich region in the central extracellular domain of NG2/HMPG (Kjellen 1991). No significant binding was seen with NG2/HMPG and type I, III, or IV (CIV) collagens (Burg 1996). The best characterised interaction is that of NG2/HMPG with CVI.

#### *Interaction of NG2/HMPG with CVI*

NG2/HMPG purified from B49 rat cell lines binds the  $\alpha 2$  chain of triple  $\alpha$  helical CVI, but not the  $\alpha 1$  chain (Burg 1996). Anti-NG2 antibodies co-precipitate NG2/HMPG and CVI from B49 rat cell lines (Stallcup 1990). Several NG2/HMPG expressing cell lines including B49 exhibit co-localisation of NG2/HMPG and CVI on the cell surface in a punctate pattern (Stallcup 1990). The distribution of CVI on

B28 rat cell lines, which do not express NG2/HMPG, is fibrillar in nature. Other ECM molecules, for example CIV, FN and LN, do not show the same degree of co-localisation with NG2/HMPG. Perturbation of NG2/HMPG distribution by antibodies results in parallel changes in CIV distribution (Stallcup 1990). This suggests that CIV and NG2/HMPG interact directly via protein-protein binding, and that NG2/HMPG may organise CIV distribution.

B28 rat cell lines and U251NG human glioma cell lines do not endogenously express NG2/HMPG. They synthesise and secrete CIV, but do not retain it at the cell surface. Transfection of these cell lines with NG2/HMPG cDNA results in expression of NG2/HMPG protein, and retention of CIV at the cell surface. CIV is distributed in a punctate pattern that co-localises with NG2/HMPG, as seen on cells that endogenously express NG2/HMPG. This effect is specific for CIV, transfection of NG2/HMPG has no effect on the distribution of FN or LN. This confirms that NG2/HMPG acts as a cell surface receptor for CIV, and that expression of NG2/HMPG is sufficient for anchorage of CIV (Nishiyama 1993).

Three different forms of NG2/HMPG exist, with MW of 275, 290 and 300 kD. Rat cell line B49 and human glioma cell line U251NG35, which express NG2/HMPG and anchor CIV, express NG2/HMPG as a 300 kD protein core. Human glioma cell line U251NG52, which expresses NG2/HMPG but fails to anchor CIV, expresses NG2/HMPG as 300 and 275 kD forms. The appearance of the 275 kD species correlates with the inability of the cells to anchor CIV. B49 cells treated with anti-NG2 antibodies, which block interaction with CIV, no longer retain CIV and produce both the 275 and 300 kD protein, suggesting that B49 cells are able to produce the 275 kD protein but do not do so under normal circumstances. These different forms are not due to alternative splicing of mRNA, so must result from post translational modification. Trypsin treatment of B49 and U251NG32 cells results in conversion of all the 300 kD protein to the 275 kD protein, which is missing the cytoplasmic domain and 64 AA of the transmembrane domain, but still remains associated with the cell surface (Nishiyama 1995).

The 290 kD form of NG2/HMPG is secreted from cells and is also missing a part of the cytoplasmic domain (Nishiyama 1995). This has also been seen with the L1 adhesion protein (Prince 1989).

Production of the 275 kD form may be regulated by interaction with CVI. The  $\alpha 3$  chain has a protease inhibitor sequence (Chu 1990) which may protect NG2/HMPG. Therefore in cells such as U251NG52, which do not retain CVI, NG2/HMPG is not protected and expressed as both intact and cleaved protein (Nishiyama 1995).

Phorbol esters PMA and PDBu, which activate PKC, and phosphatase inhibitor okadaic acid cause a shift towards the production of the 275 kD protein and a decrease in the 300 kD protein in a dose dependent manner. Treatment of the cells with the PKC inhibitor staurosporine results in a quantitative decrease in the 275 kD form and an increase in the 300 kD form. These events occur within minutes (Nishiyama 1995). PKC may activate *de novo* synthesis of a protease. Phorbol esters stimulate stromelysin and collagenase activity to cleave aggrecan at asparagine-phenylalanine sites (Frisch 1987) of which there are two in NG2/HMPG. Phorbol esters also activate elastase which cleaves at alanine-valine (Pandiella 1991) of which there are two in NG2/HMPG. NG2/HMPG contains numerous lysine-arginine sites at which trypsin is known to act (Bernfield 1992).

NG2/HMPG has been reported to act as a CVI receptor in other cell types. In corneal fibroblasts and undifferentiated peri-ocular mesenchyme *in vivo*, and in mesenchymal cells grown *in vitro* on CVI, NG2/HMPG and  $\alpha 3\beta 1$  localise to focal contacts and interact with CVI (Doane 1998).

#### *Mechanism of NG2/HMPG Interaction with CVI*

Precise characterisation of the domains of NG2/HMPG responsible for its collagen binding properties was achieved by electron microscopy of recombinant NG2/HMPG (Tillet 1997). NG2/HMPG comprises two cysteine-rich domains joined by a cysteine-free domain. Domains 1 and 3 exhibit a globular conformation stabilised by disulphide bonds and are separated by the extended cysteine free second domain. Electron microscopy of NG2/HMPG-CVI mixtures shows that the collagenous triple



helical domain aligns with the central rod like domain 2 of NG2/HMPG. Fragments of NG2/HMPG used in binding assays confirm that domain 2 is the site of attachment for CVI. This domain alone supports CVI binding and proteins lacking this segment do not. Domain 2 is also responsible for CS GAG attachment. CS GAGs are not thought to have a direct role in CVI binding, although NG2/HMPG synthesised without the addition of CS is unable to bind CVI. This suggests that GAG chains may modulate the folding of NG2/HMPG into a conformation able to interact with extracellular ligands (Tillet 1997). **FIGURE 1.3a.**

A series of NG2/HMPG variants, with deletions throughout the entire coding sequence of the protein, were expressed in B28 rat cell lines, to study the effect of the specific mutations on the ability to interact with CVI. All but three of the mutant NG2/HMPG molecules enable cells to anchor CVI at the cell surface. Analysis of the deleted sequences of the three revealed that a 469 amino acid segment between residues 575 and 1044 of NG2/HMPG protein core are essential for CVI binding. These are found in the extracellular central domain 2 of NG2/HMPG (Burg 1997).

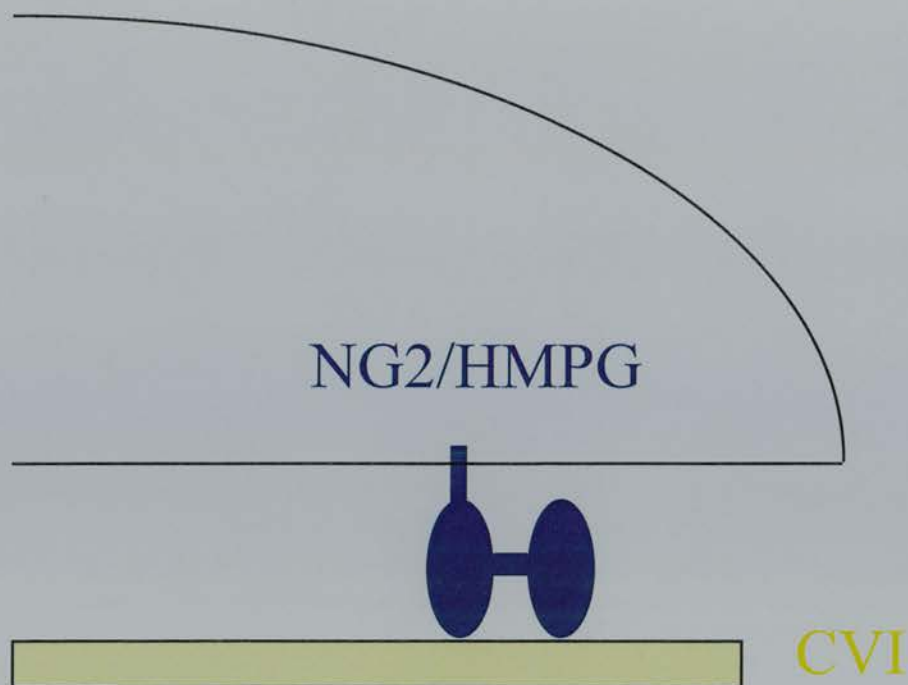
Amino acids 750-850 of domain 2 of NG2/HMPG are predicted to form a similar type of secondary structure conformation to the leucine-rich repeats of decorin and related PGs, which consist of an alternating  $\alpha$ -helical and  $\beta$ -turn units that form an open horseshoe-like structure in which the collagen helices are proposed to bind (Scott 1996). **FIGURE 1.3b.**

#### *Functional Relevance of NG2/HMPG Interaction with CVI*

CVI is found in most if not all connective tissues, as a heterotrimer. The chains form a small triple helical domain which separates the large globular domains at the N and C terminus. These trimers form large aggregates stabilised by disulphide bonds (Ayad 1994).

O-2A cells do not express CVI, but areas in the developing rat do co-express CVI and NG2/HMPG, for example some blood vessels, spinal column, intestines and the cartilaginous structures of the head. This suggests that the interaction between NG2/HMPG and CVI demonstrated *in vitro* may have some relevance *in vivo*. (Stallcup 1990)

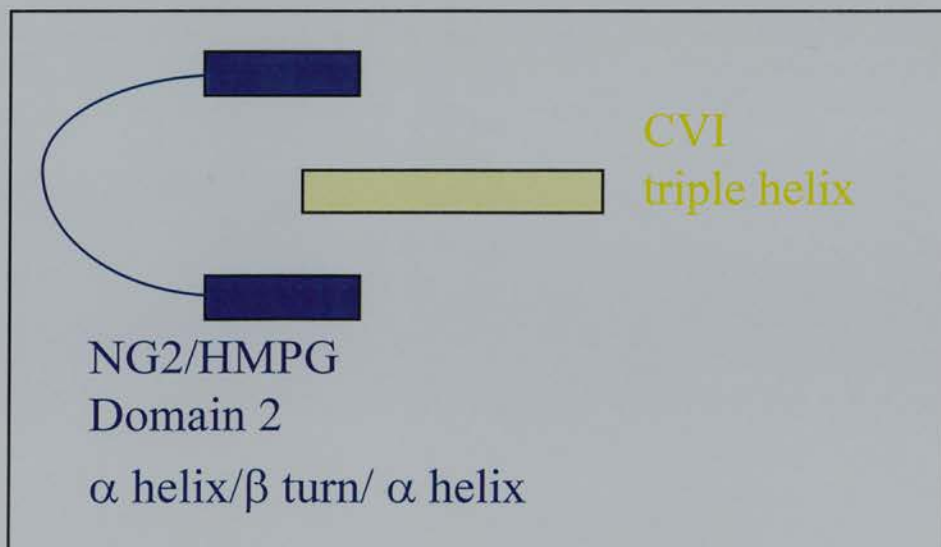




**Figure 1.3.a**

**Interaction of NG2/HMPG with CVI.**

NG2/HMPG at the cell surface directly interacts with CVI in the ECM.



**Figure 1.3.b**

**Interaction of NG2/HMPG with CVI (detail).**

The CVI triple helix inserts into the horse shoe like configuration of domain 2 of the NG2/HMPG molecule.

### *Cell adhesion*

CVI is localised to the pericellular matrix and basement membranes, close to the cell surface in a number of cells, including developing nerves (Keene 1988). It interacts with many ECM proteins including HA (McDevitt 1991, Kielty 1992), TN (Faissner 1990), fibrillar collagens I and II (Hedbom 1993, Bonaldo 1989, Bidanset 1990, Keene 1988), type XIV collagen (Brown 1993), decorin (Hedbom 1993) and fibromodulin (Vogel 1984, Bonaldo 1989), as well as with cell surface receptors such  $\beta 1$  integrins (Aumailley 1989),  $\alpha 1$ ,  $\alpha 2$  and  $\alpha 3$  integrins (Pfaff 1993, Doane 1992, Wayner 1987), and PGs including CSPGs (Bray 1990) and CD44 (Gallatin 1986, Carter 1982). CVI therefore is important in anchoring cells to the ECM, in a manner similar to the points of attachment provided by the interaction of FN with collagens and integrins (Ruoslahti 1988).

These points of adhesion are also involved in signal transduction between the ECM and the inside of the cell. Interactions between CVI and NG2/HMPG may provide alternative means of transmitting information across the cell membrane, raising the possibility that these molecules are important not only in structural roles (organisation of the ECM, binding of cells to the matrix and determination of cell morphology) but also in modulation of cellular processes including proliferation, migration and differentiation.

### *Cell migration*

NG2/HMPG negative rat B28 cell lines and human U251NG glioma cell lines can migrate towards CVI, suggesting that, in the absence of NG2/HMPG, these cells still have CVI recognition molecules, which may include  $\alpha 1\beta 1$ ,  $\alpha 2\beta 1$  integrins (Pfaff 1993, Doane 1992) and CD44 (Carter 1982). However, NG2/HMPG expression in these cells specifically enhance this migratory response. This stimulation requires the same region of NG2/HMPG that is responsible for interaction with CVI, AA 575-1044. Deletion mutants missing this domain fail to produce a similar enhanced migration (Burg 1997). There are a number of mechanisms by which this may occur, NG2/HMPG may act to alter the actin CS conformation resulting in increased cellular motility, as NG2/HMPG has been shown to interact with actin in actively

spreading glioma cells (Lin 1996a,b). Alternatively NG2/HMPG may alter the interaction of CVI with its other receptors in such a way that potentiates cell motility, for example by modulating CVI interaction with integrins in the same way that NG2/HMPG does with  $\alpha 4 \beta 1$  with melanoma cell spreading on FN (Iida 1995). CVI is found along neural migratory pathways in developing CNS (Perris 1993). Neural crest cells bind to, and migrate, along CVI *in vitro* (Perris 1993). Glial cells (Han 1995) and uveal melanoma cells (Daniels 1996) that produce more CVI are more invasive than those that do not, suggesting a correlation between CVI expression and malignancy.

#### *Tumour cell invasion and proliferation*

NG2/HMPG is expressed in the most malignant of glioma phenotypes (Schrappe 1991). During transformation glioma ECM composition changes, including increased expression of TN, HA and CVI (McKeever 1989, Chintala 1996), which is thought to stimulate the spread of cells throughout the brain, possibly by interacting with CIV in endothelial basement membrane (Kuo 1997). NG2/HMPG binds both TN and CVI, so may assist in promoting interaction with the ECM under these circumstances, indicating a possible role for NG2/HMPG in tumour cell invasion and migration of neoplastic cells (Nishiyama 1993).

Platelet derived growth factor (PDGF) and receptor are also upregulated in malignant glioma cells (Westermarck 1995). CVI binds PDGF to form a functional complex (Somasundaram 1996). NG2/HMPG has been shown to potentiate smooth muscle and O-2A progenitor cell response to PDGF by interacting with the PDGF $\alpha$  receptor (PDGF $\alpha$ -R), it may do the same in glioma cells. This could be of interest, as NG2/HMPG and CVI co-localise in some tissues in developing rat cartilage, where NG2/HMPG-CVI interactions may be important during development but less so after maturation, at which time NG2/HMPG expression is down regulated (Nishiyama 1991a).

### **1.7.3. Modulation of human melanoma cell interaction with FN by NG2/HMPG**

FN interacts with many ligands, including other FN molecules, ECM proteins and cell surface receptors. Integrins mediate cell adhesion to FN (Akiyama 1996, Hynes 1987), leading to the activation of numerous intracellular responses, including cytoskeletal reorganisation and tyrosine phosphorylation (Clark 1995, Lafrenie 1996). Integrins are important in modulating tumour cell migration and adhesion (Albeda 1990, Hynes 1992). Several PG also interact with FN (Hook 1984), for example syndecan (Gallagher 1989) and CSPG expressed by chick embryo fibroblasts (Yamagata 1989), although the molecular nature of the downstream events are less well characterised.

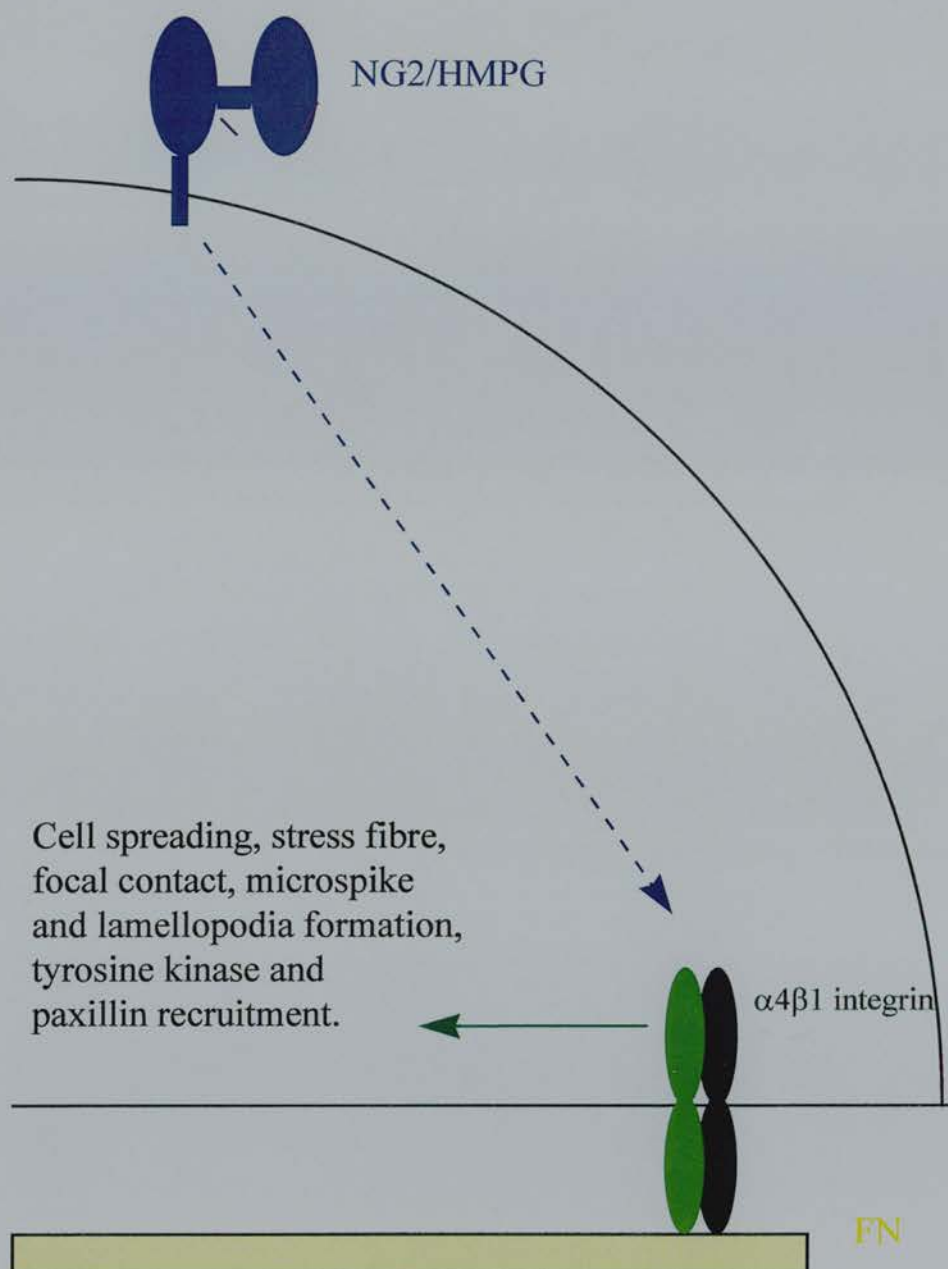
NG2/HMPG and FN co-localise during initial condensation of rat chondrocytes (Nishiyama 1991a). Anti-HMPG antibody AMF-6 inhibits melanoma cell migration along FN concentration gradients, a process by which cells adhere to, and invade the vascular endothelium *in vivo*. It has no effect on cell adhesion, suggesting that NG2/HMPG is not involved this process, but in the chemotactic response and kinetic motility of melanoma cells (DeVries 1986).

A 33 kD fragment from the C terminus of FN promotes mouse melanoma adhesion and spreading (McCarthy 1990). Melanoma cell adhesion to this fragment can be completely blocked by anti- $\alpha 4\beta 1$  antibodies and inhibited 55% by agents that block CSPG function by removing CS GAGs. This suggests that melanoma cell adhesion to this FN fragment involves specific integrin subunits, and is partially mediated by CS GAG chains (Iida 1992). Melanoma cells adhere to anti-HMPG antibody 9.2.27, FN, a FN peptide (CS1), laminin, CIV, and chimeric substrates comprising LN and 9.2.27 or CIV and 9.2.27 (Iida 1995). However, no cell spreading, focal contact and stress fibre formation, cell flattening, microspike and lamellopodia production, or paxillin recruitment to focal contacts is observed unless melanoma cells are grown on a chimeric substrate of both FN/CS1 and 9.2.27.  $\alpha 4\beta 1$ /NG2/HMPG-stimulated spreading on CS1 and 9.2.27 is sensitive to tyrosine kinase inhibitors, and two proteins of molecular weight 120 and 130 kD, immunologically unrelated to p125FAK, are phosphorylated on tyrosine residues.

This does not occur during melanoma adhesion (Iida 1995). This suggests that both NG2/HMPG and  $\alpha 4\beta 1$  must be stimulated to promote these events.

*Mechanism of Modulation of Human Melanoma Cell Interaction with FN by NG2/HMPG*

NG2/HMPG and  $\alpha 4\beta 1$  co-ordinately mediate melanoma cell interaction with FN. However, they need not be in close proximity on the cell surface to stimulate melanoma cell spreading and focal contact formation.  $\alpha 4\beta 1$  must be in contact with the substrate, but NG2/HMPG need not. Melanoma cells already adherent on CS1 can be stimulated to spread and form focal contacts by addition of 9.2.27 coated beads, however cells adherent on 9.2.27 can not be stimulated to spread by CS1 coated beads. This suggests that NG2/HMPG may modify  $\alpha 4\beta 1$  binding to FN by an inside out mechanism (Iida 1995). This has been described in other cell types; integrin conformation is altered by signals within the cell involving the cytoplasmic domains of the integrin, modulating the affinity of the integrin for its ligand (Hynes 1992). **FIGURE 1.4.**



**Figure 1.4.**

**Role of NG2/HMPG in mediating human melanoma cell interaction with FN.**

**NG2/HMPG indirectly modulates melanoma cell adhesion to FN.**

**By inside out signalling NG2/HMPG alters the affinity of  $\alpha 4 \beta 1$  integrin for FN. This results in cell spreading, focal contact formation, microspike and lamellopodia extension and tyrosine kinase recruitment.**



*Functional Relevance of Modulation of Human Melanoma Cell Interaction with FN by NG2/HMPG*

Integrins are important in mediating cell adhesion to ECM, but they do not alone deliver the entire range of signals required by a cell to undergo associated processes such as spreading and focal contact formation. Chinese hamster ovary cells that are HS deficient form fewer focal contacts than normal cells (Lebaron 1988). Both integrin and PG binding fragments of FN are required for fibroblast focal contact formation (Woods 1992). Syndecan regulates integrin function via inside out signals involving PKC (Woods 1992, 1993, 1994). NG2/HMPG contains three threonines which are potential targets for PKC phosphorylation (Nishiyama 1991b), however there is no sequence homology between NG2/HMPG and  $\beta 1$  integrin cytoplasmic domains as has been observed for syndecan (Argraves 1987). Alternative means of NG2/HMPG regulating integrin interaction with FN may involve tyrosine phosphorylation. Tyrosine phosphorylation of  $\beta 1$  in transformed cells leads to a decrease in its affinity for FN and talin (Hynes 1992). Two proteins are specifically phosphorylated during HMPG and  $\alpha 4\beta 1$ -stimulated spreading, however their identity is unknown (Iida 1995). NG2/HMPG may act as a co-receptor for FN, co-ordinating cell adhesion and signal transduction.

*Comparison of NG2/HMPG Interaction with CVI and FN*

NG2/HMPG expressed in B49 rat cell lines interacts directly with CVI. In contrast NG2/HMPG expressed by human melanoma cells indirectly affects cell adhesion by modulating  $\beta 1$  integrin interaction with FN. Evidence suggests that cell surface PGs and integrins exert a co-ordinated effect on matrix functions, although there is no difference in the distribution of  $\beta 1$  integrin in control or NG2/HMPG transfected rat cell lines, indicating that NG2/HMPG-CVI interaction is  $\beta 1$ -independent. This suggests that NG2/HMPG mediates interactions with different ECM proteins via at least two different pathways.

#### **1.7.4. Induction of HMPG expression by ECM**

The relationship between NG2/HMPG expression and cell-ECM interaction has been studied (Rettig 1986). Human chromosomes retained in rodent-human melanoma hybrid clones were determined by karyotype and cell surface marker analysis. Expression of NG2/HMPG was mapped to chromosome 15. NG2/HMPG expression can be induced by fusion of specific cell types which retain chromosome 15, for example, non NG2/HMPG-expressing human kidney epithelial cells and mouse fibroblasts. In contrast, human kidney cells fused with mouse renal carcinoma cells did not express NG2/HMPG, even though chromosome 15 is retained. NG2/HMPG expression can also be induced by the ECM. Human and mouse neuroblastoma chromosome 15-containing hybrids grown on plastic are loosely attached, appear rounded with extended processes, and do not express NG2/HMPG or become confluent. However on plates coated with ECM derived from bovine corneal epithelial cells, NG2/HMPG expression is induced, cells adhere strongly, spread and become confluent. NG2/HMPG-negative cell lines of epithelial and hematopoietic origin did not show induced NG2/HMPG expression, and SV13 and F23 proteins, also encoded for by chromosome 15, show no change in expression, suggesting that this effect is specific for NG2/HMPG and target cell type. NG2/HMPG is the first antigen shown to be regulated in this way. Expression of NG2/HMPG specifically induced by the presence of the ECM is accompanied by changes in cell morphology and adhesive ability. This suggests alterations in multiple pathways and genes, and that both intrinsic and extrinsic signals, provided by the rodent partner and the ECM respectively, can induce NG2/HMPG expression in previously non-expressing cells.

#### **1.7.5. Tumour metastasis**

Tumour invasion and metastasis requires cell detachment from the primary tumour and relocation to secondary tissue sites where colonies of cells are established (Cifone 1980). Inhibitors of the cell surface receptors  $\alpha 2\beta 1$ , CD44 and NG2/HMPG, which are expressed at high levels in melanoma cells, inhibit melanoma invasion through basement membranes. CD44 and  $\alpha 2\beta 1$  do this by mediating interactions with the CIV component of the membranes, however NG2/HMPG does not and



therefore must modulate invasion in an alternative manner (Knutson 1996). Antibodies against NG2/HMPG inhibit melanoma cell colony-forming ability (Harper 1983), tumour growth in mice (Bumol 1983), and cell spreading on bovine aorta endothelial basement membranes, but have minimal effects on cell adhesion (Bumol 1984, DeVries 1986). This suggests that NG2/HMPG is important in melanoma cell spreading and stabilising cell substrate interactions, and that anti-HMPG antibodies inhibit processes essential for tumour growth by interfering with cell-cell/substratum interactions. Anti-HMPG antibody 155.8 inhibits adhesion of melanoma cells to plastic, FN, collagen, and collagen/FN substrates (Harper 1984), suggesting that NG2/HMPG is important in interaction with these ECM molecules

#### **1.7.6. Functions implicated by NG2/HMPG primary structure**

Internal repeats in the sequence of NG2/HMPG are similar to those found in chick N-cadherin (Hatta 1988), the highest similarity being in a proposed calcium-binding site (Hatta 1988, Ringwald 1987). Cadherins are calcium dependent cell adhesion molecules. They are important in mediating cell-cell interactions via homo/heterotypic extracellular interactions and intracellular interactions with catenin molecules which link them to the actin cytoskeleton to construct ordered multicellular tissues and structures. Cadherins are important in the formation of CNS architecture (Redies 1996), in axonal guidance and in conveying adhesive flexibility required during tumour cell invasion and metastasis (Bussemakers 1996). NG2/HMPG may have a similar function.

#### ***SUMMARY***

The localisation of NG2/HMPG at the cell surface indicates a role in cell interaction with the ECM. In rat cell lines NG2/HMPG binds directly to a number of ECM proteins, the best studied of which is CVI. NG2/HMPG modulates the interaction of human melanoma cells indirectly, by outside in signalling resulting in an increase in adhesion of integrins to FN. NG2/HMPG appears to play a key role in the adhesion of both normal and transformed cells to ECM components, and also in other cell functions including spreading, focal contact formation and migration.

## **1.8. FUNCTION OF NG2/HMPG AS A SIGNAL TRANSDUCTION MOLECULE**

### **1.8.1. Interaction of NG2/HMPG with actin in rat cell lines**

As a membrane spanning protein NG2/HMPG is able to interact with molecules on both the inside and outside of cells. In rat cell lines NG2/HMPG is expressed in a punctate pattern on the cell surface in ordered linear arrays, which co-localise with actin stress fibres. This is most obvious in extremely flattened cell lines, for example B111. Disruption of the cytoskeleton by cytochalasin D causes NG2/HMPG to aggregate into patches, and results in an increase in the amount of NG2/HMPG released from the cells. No co-localisation occurs with tubulin or vimentin, suggesting that NG2/HMPG interacts specifically with actin (Lin 1996a).

Transfection of B28 rat cell lines, which do not endogenously express NG2/HMPG, with NG2/HMPG cDNA results in a punctate distribution similar to B111 cells, indicating that anchorage of NG2/HMPG to actin occurs via a mechanism not specific to endogenous NG2/HMPG expressing cells (Lin 1996a).

Other molecules which interact with actin in B28 cells, endogenous  $\alpha 5 \beta 1$  and CD44, and transfected L1, also form linear arrays on the cell surface, which co-localise with stress fibres. The cytoplasmic domains of these proteins and NG2/HMPG show no similarity, indicating that they interact with actin via different linker molecules (Lin 1996a).

B28 cells treated with colchicine undergo retraction of the cytoplasm, loss of flattened morphology and retention of resistant tendrils. These tendrils express NG2/HMPG,  $\alpha 5 \beta 1$  and actin, but not myosin, CD44 and L1. Chimeras of NG2/HMPG cytoplasmic domain and L1 ectodomain localise as full length NG2/HMPG in the tendril, whereas expression of chimeras containing the cytoplasmic domain of L1 and the ectodomain of NG2/HMPG is restricted to the soma. This suggests that the specific sequence of the intracellular domain determines whether a molecule can interact with actin in the soma, or in the colchicine resistant tendrils (Lin 1996a).

Transfected B28 cell cultures not treated with colchicine often contain a small population of cells which have a naturally rounded morphology in preparation for division, or to respond to changes in the local environment (Lin 1996a). These cells retain NG2/HMPG-positive tendrils which function as retraction fibres (Cramer 1993), and remain attached to the substratum during mitosis to provide a framework for resulting cells to adhere to the substratum and rapidly spread (Lin 1996a).

In actively spreading B28 cells, integrin mediated binding to FN promotes stress fibre formation with which NG2/HMPG co-localises. Electrostatic attachment to poly-L-lysine (PLL) results in cells that are less flattened with no stress fibres, but a dense array of actin containing projections radiating from the soma, which express NG2/HMPG at the ends. The actin binding protein fascin is expressed by similar radial projections in cells grown on PLL (Adams 1995). However the NG2/HMPG positive projections are individual self contained units of membrane and cytoplasm i.e. filopodia, which are distinct from, and extend beyond, the area occupied by fascin containing lamellopodia sheets of membrane. NG2/HMPG-positive projections contain  $\alpha 5 \beta 1$  integrin, but not L1, myosin or focal adhesion plaque markers vinculin or phosphotyrosine, which are present in fascin positive projections. B28 cells spreading and migrating on PLL are highly polarised with NG2/HMPG-positive filopodia at one pole and fascin-positive lamellopodia at the other. Migration occurs in the direction of the lamellopodia (Lin 1996b).

The cytoplasmic domain of NG2/HMPG is responsible for targeting the protein to the projections, but the exact sequence required for this specific targeting is unknown (Lin 1996b).

The extent of NG2/HMPG's involvement in cell spreading is unclear. Some NG2/HMPG-positive cells do not form filopodia when grown on PLL, and untransfected B28 cells still form these projections. For most cell lines tested, a substrate was identified that promoted NG2/HMPG positive filopodia formation, for example U251NG35 human glioma cell lines require PLL and FCS. This suggests that NG2/HMPG expression is insufficient to cause filopodia formation, but in appropriate conditions that allow formation, NG2/HMPG is preferentially associated with them. It is unclear whether these processes are exploratory filopodia or

retraction fibres. The fact that they are expressed by rounded up cells treated with colchicine, and at the trailing edges of migrating cell, suggests that they provide a more supportive function in allowing cells to release from the substrate and migrate. However, they are also seen on freshly plated B28 cells grown on PLL, indicating a function in extending cell membrane over an area not previously occupied by the cell, i.e. filopodia. NG2/HMPG's role appears to be indirect in the formation of these projections, and associated with integrins (Lin 1996b).

#### *Functional Relevance of NG2/HMPG Interaction with Actin*

Actin is one of the most abundant proteins in the cell. It forms part of the cytoskeleton, which is involved in many cellular functions, including shape regulation, migration, mitosis and anchorage dependent growth. Actin forms an integral part of focal adhesion plaques, which are essential in the adhesion of cells to ECM, and transmission of signals between the inside and outside of cells. This suggests that the association of actin and NG2/HMPG may participate in such cellular functions.

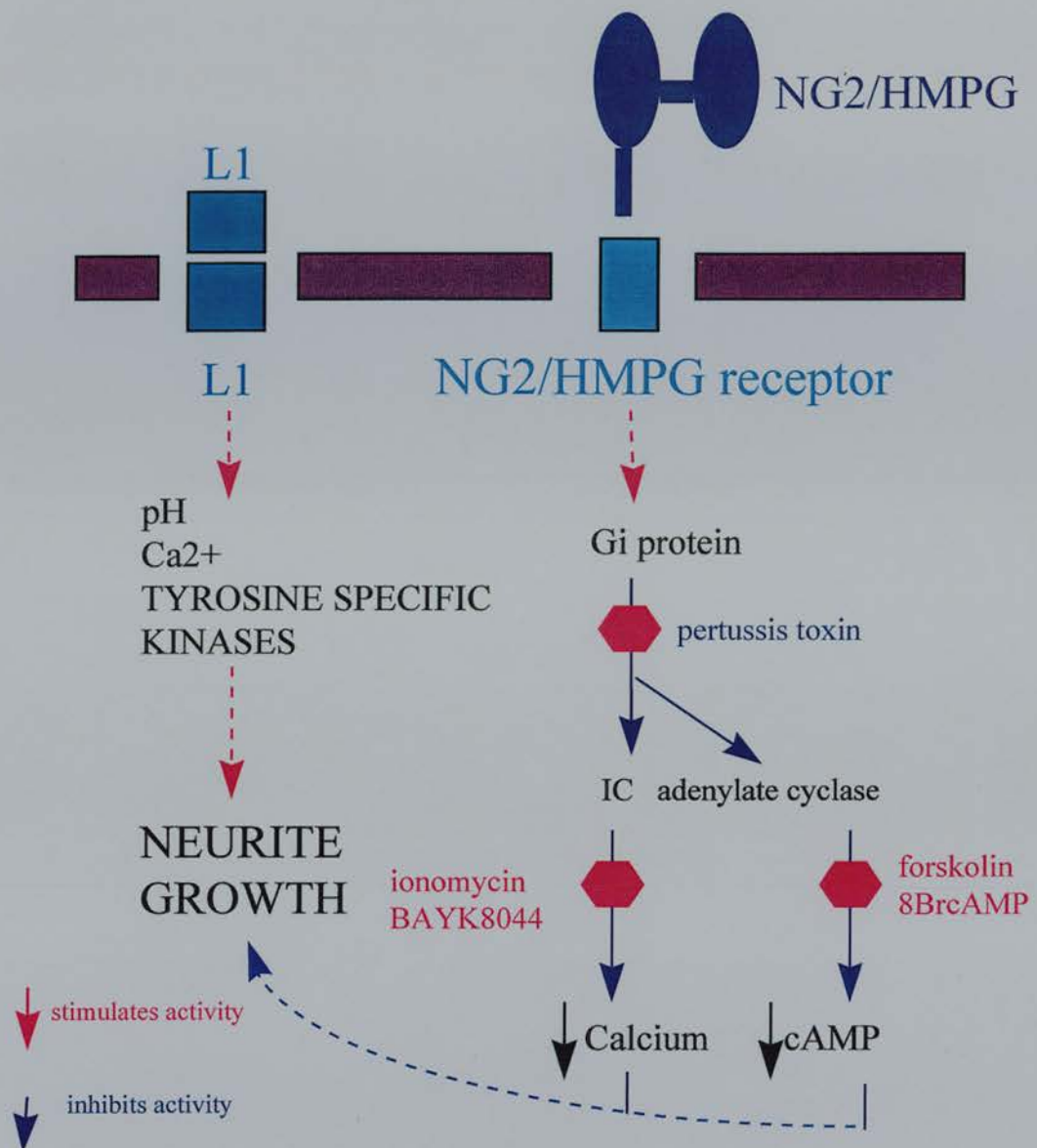
Changes occur in the melanoma cell cytoskeleton, the amount of actin increases in parallel with an increase in migration and metastatic ability (Gabbiani 1979).  $\alpha$ -actinin and  $\beta$ 1 integrin expression increases in focal adhesions in melanoma cells, which correlates with an increase in migration rate (Byers 1992). NG2/HMPG is thought to modulate  $\beta$ 1 integrin interaction with FN in melanoma cells (Iida 1995), and may do this by participating in focal adhesion binding to actin. NG2/HMPG plays a role in melanoma cell spreading on FN, NG2/HMPG in rats may also have a role in cell spreading. In rat cells actin and NG2/HMPG protein and mRNA expression decreases throughout development with increasing age (Schmitt 1977, Nishiyama 1991a).

### 1.8.2. Inhibition of rat neurite growth by NG2/HMPG

NG2/HMPG is involved in the interactions needed during nervous system development (Dou 1994). L1, a cell adhesion glycoprotein abundant in the developing CNS, supports neuronal cell attachment and promotes elongation *in vitro*. The core protein of NG2/HMPG purified from B49 rat cell lines inhibits rat neurite growth on L1. Neurites grown on borders of NG2/HMPG and L1 avoid NG2/HMPG. L1-L1 interactions are important in CNS tissue formation (Appel 1993) and L1 activation of neurite growth involves several intracellular second message systems including regulation of intracellular pH, calcium concentration, and tyrosine kinase activity (Williams 1994), which could be modulated by NG2/HMPG. Developing rat neurites do not express NG2/HMPG, but do express a specific, high affinity 280 kD cell surface receptor for NG2/HMPG whose identity is unknown (Dou 1997). NG2/HMPG acts by binding to this receptor and altering intracellular pathways, which results in a change in neural cell growth capacity. Pharmacological analysis reveals that this occurs by G protein-mediated closure of calcium channels and inhibition of adenylate cyclase, decreasing intracellular calcium and cAMP levels and results in inhibition of neurite growth. Inhibitors of these intracellular signalling processes reverse inhibition of neurite growth on L1 caused by NG2/HMPG, but have no effect on neurite growth on L1 alone (Dou 1997) (**FIGURE 1.5.**). This may occur by decreasing calcium levels, which lowers the stability of the CS in the neurites (Kater 1994) and cAMP directly enhancing neurite outgrowth, as it does from chick embryonic cerebral implants (Wong 1991).

Other core proteins of rat CNS CSPGs inhibit neurite growth (Cole 1991). Developing CNS anatomy suggests that NG2/HMPG-expressing cells help keep growing parallel fibres stacked in the molecular layer ensuring they do not stray out into inappropriate areas (Altman 1972).





**Figure 1.5.**

**Inhibition of rat neurite growth by NG2/HMPG.**

L1-L1 interaction stimulates neurite growth. Modification of intracellular pH, calcium concentration and tyrosine kinase activity are signalling events implicated in the pathway resulting in cell elongation. NG2/HMPG binding to neurite cell surface receptors activates Gi protein inhibition of adenylate cyclase and calcium ion channels, resulting in decreased concentrations of intracellular calcium and cAMP, which ultimately leads to inhibition of neurite growth. Inhibitors of Gi proteins (pertussis toxin) and agents which increase calcium (ionomycin, BAYK8044) or cAMP (forskolin, 8BrcAMP) block NG2/HMPG mediated inhibition of neurite growth.

### *The role of NG2/HMPG in the response to CNS injury*

Injury to the mature rat CNS results in a transient increase in the number of NG2/HMPG-positive cells, concomitant with an increase in the level of NG2/HMPG expression by individual cells and DNA synthesis. NG2/HMPG may be involved in the inhibition of axonal re-growth, which is limited after injury (Levine 1994).

Sections of adult rat brain contain numerous O-2A NG2/HMPG-expressing cells which interact with microglia. In mice with inflammatory lesions in the brain and de-myelination, for example in conditions such as autoimmune encephalomyelitis, NG2/HMPG-positive cells increase in number immediately around the affected area and become closer to microglia, suggesting a functional interaction (Nishiyama 1997).

Antibody induced de-myelination in rat spinal cord also results in an increase in the number of NG2/HMPG-expressing cells in the lesion area, which persist until re-myelination. This may be important in pathological conditions caused by failure to re-myelinate such as multiple sclerosis (Keirstead 1998). O-2A NG2/HMPG positive progenitor cells express PDGF $\alpha$ -R *in vivo*, suggesting that these cells can respond to both types of growth factor, which may account for re-population of de-myelinated lesions by these cells during re-myelination (Redwine 1997).

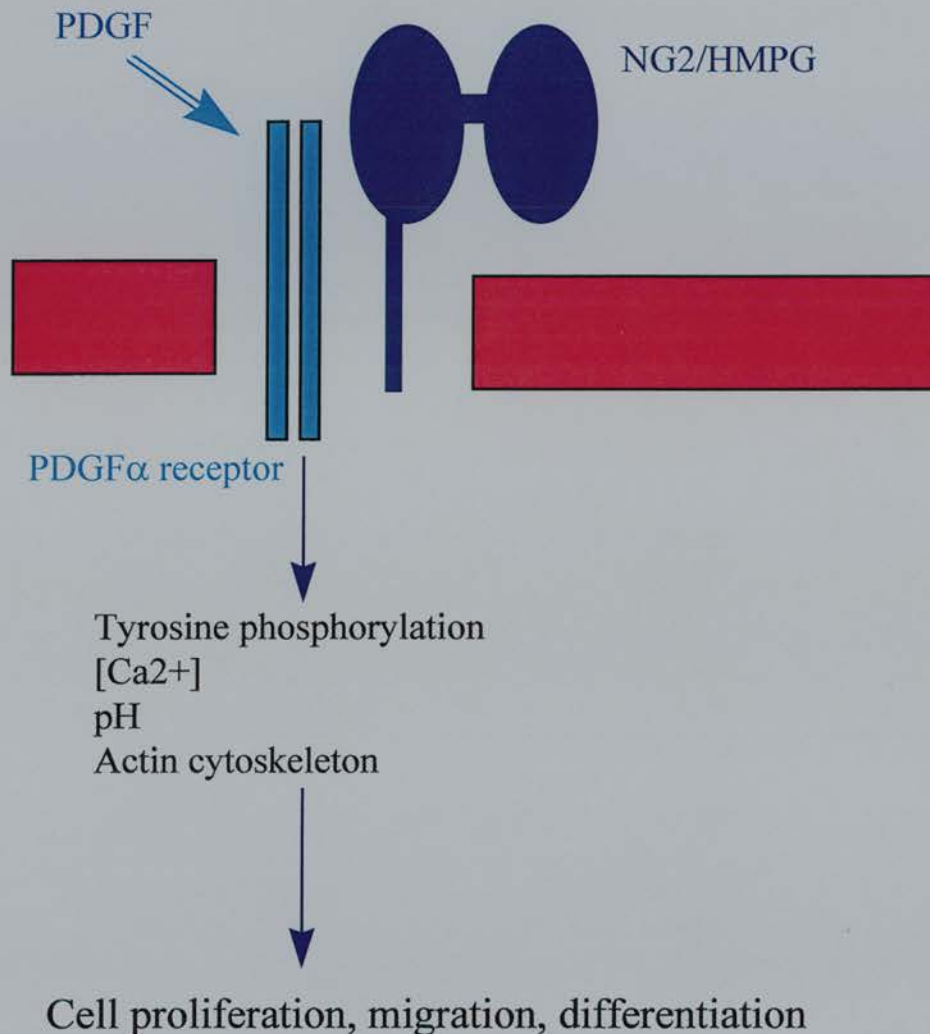
#### **1.8.3. Interaction of NG2/HMPG with PDGF $\alpha$ receptor**

NG2/HMPG and PDGF $\alpha$ -R are expressed co-ordinately during early stages of rat O-2A cell development. *In vivo* in the embryo the number of blood vessels and glial cells expressing NG2/HMPG and PDGF $\alpha$ -R, and degree of co-localisation to cell bodies and processes, increases until peaking at postnatal day 10. After this time expression and co-localisation of NG2/HMPG and PDGF $\alpha$ -R declines; PDGF $\alpha$ -R expression is restricted to the cell bodies, while NG2/HMPG is also found on cell processes. In the mature brain both continue to be expressed with this distribution (Nishiyama 1996a). This closely co-ordinated expression also occurs *in vitro* where the expression of NG2/HMPG coincides with the ability of O-2A cells to respond to PDGF (Nishiyama 1996b). O-2A cells initially express NG2/HMPG and PDGF $\alpha$ -R in a co-localised manner. After differentiation into mature

oligodendrocytes, which do not express NG2/HMPG but continue to express PDGF $\alpha$ -R (Raff 1988), or differentiation into type 2 astrocytes (Raff 1983) which do not express PDGF $\alpha$ -R but continue to express NG2/HMPG, neither cell type responds to PDGF (Hart 1992). O-2A cells treated with PDGF are maintained as O-2A precursor cells, with a delay in the down regulation of NG2/HMPG and PDGF $\alpha$ -R, an increase in the number of cells that express both NG2/HMPG and PDGF $\alpha$ -R, a higher degree of co-localisation, and a quantifiable increase in the amount of the two molecules expressed by each cell. Antibodies against PDGF $\alpha$ -R precipitate both NG2/HMPG and the receptor from O-2A cell lysates, suggesting the two proteins form a molecular complex or part of a complex. When the expression of NG2/HMPG is interfered with by incubation of cells with anti-NG2 antibody, the amounts of both protein decrease, do not co-localise and occur in patches. Cells also no longer proliferate as much in response to PDGF. This indicates that correct expression of NG2/HMPG and PDGF $\alpha$ -R on the cell is required for optimal response to PDGF (Nishiyama 1996a). **FIGURE 1.6.**

Developing and adult rat vascular smooth muscle cells also express NG2/HMPG, PDGF and PDGF $\alpha$ -R. DNA synthesis and migration of primary cultures of postnatal rat aortic smooth muscle is stimulated by the two forms of PDGF, AA and BB. Stimulation by PDGF AA, but not BB, is inhibited by incubation with anti-NG2 antibody, suggesting a specific response mediated by the interaction of PDGF AA, the  $\alpha$  receptor and NG2/HMPG (Grako 1995).





**Figure 1.6**

**Interaction of NG2/HMPG and PDGF $\alpha$  R.**

**NG2/HMPG interaction with PDGF $\alpha$ -R at the cell membrane results in optimal cell response to PDGF, which involves tyrosine phosphorylation, changes in intracellular calcium concentration and pH, and actin cytoskeleton re-organisation. These events stimulate cellular functions including proliferation, migration and differentiation.**

### *Mechanism of Interaction of NG2/HMPG with PDGF $\alpha$ -R*

Correct expression of NG2/HMPG on the cell surface leads to an interaction between NG2/HMPG and PDGF $\alpha$ -R, enabling the cells to respond optimally to PDGF during development. NG2/HMPG may act by presenting an activated form of PDGF to the receptor. It may alter receptor conformation to activate receptor ligand interaction, or affect down stream signalling. After development the inability of the two molecules to form a functional complex may be responsible for the lack of response to PDGF, either due to down regulation of NG2/HMPG expression or of other molecules which may be required. Expression of NG2/HMPG is not down regulated in aortic smooth muscle cells, where it may also play a role in mature cells in injury or disease responses.

### *Functional Relevance of Interaction of NG2/HMPG with PDGF $\alpha$ -R*

PDGF receptors are cell surface single span transmembrane tyrosine kinases (Matsui 1989), which dimerise upon ligand binding (Heldin 1995) and initiate a number of responses, including intracellular calcium fluxes, cytoplasmic pH changes, rearrangement of actin stress fibres, and membrane ruffling as well as long term responses including migration, proliferation and differentiation (Heldin 1995). PDGF and both types of receptor are widely expressed in neural and glial cells of the central and peripheral nervous system (Valenzuela 1994), where they play an important role in neuronal and glial cell development as well as in the mature and pathological CNS.

NG2/HMPG modulation of PDGF AA responses in postnatal rat cells may be significant as this is the period during development when the smooth muscle cells double in number due to the autocrine activities of PDGF (Schwartz 1990). NG2/HMPG is also expressed in adult rat aortic smooth muscle cells, therefore it may also be important in adult cells in the modulation of response to PDGF, for example after tissue damage such as arterial injury or atherosclerosis when smooth muscle cells migrate to and proliferate in the affected areas. Unpublished studies have revealed that NG2/HMPG is expressed by rat and human proliferating smooth muscle cells in atherosclerotic arteries (Grako 1995).



Many transformed cells synthesise PDGF and its receptor (Heldin 1995), which may participate in the adhesion and spreading process of melanoma cells to FN, in concert with NG2/HMPG and  $\alpha 4\beta 1$ , possibly by tyrosine phosphorylation.

NG2/HMPG is expressed in a number of different cell types, many of which are developing and proliferating. Down regulation occurs once the cells mature and proliferation decreases, for example the rat CNS and limb (Levine 1987a, Nishiyama 1991a). NG2/HMPG is also expressed pathological states of stimulated proliferation, for example human melanoma and rat chondrosarcoma (Bumol 1982, Leger 1991). This indicates that NG2/HMPG may regulate cell proliferation by modulating the action of PDGF via interaction with PDGF $\alpha$ -R.

#### **1.8.4. Functions implicated by NG2/HMPG primary structure**

##### *Protein kinase C phosphorylation sites.*

The cytoplasmic domain of NG2/HMPG contains three threonine residues whose surrounding sequences conform to the motif for PKC phosphorylation sites. The family of PKC enzymes alter the function of target proteins by phosphorylating specific serine/threonine residues, resulting in regulation of cell functions including proliferation and differentiation (Nishizuka 1995). The major signalling pathways in melanocytes involve PKC. This is altered in transformed cells, PKC  $\beta$  isoforms are lost (Yamanishi 1994), and PKC activity directly correlates with the chemotactic and invasive abilities of melanoma cells (La Porta 1997, Mapelli 1994). Phorbol esters are potent tumour promoters which act by prolonging the activation of PKC, resulting in the phosphorylation of  $\alpha 3\beta 1$  integrins (Dumont 1994). Melanoma adhesion to, and spreading on, FN requires activation of PKC (Smith T.W 1996b, Tang 1995) and expression of  $\alpha 4\beta 1$  integrin (Saini 1997). PKC appears to be involved in promoting melanoma metastasis, possibly by regulating adhesion to FN in concert with integrins.

Processing of NG2/HMPG to a 275 kD sized protein can be increased by activation of PKC and decreased by inhibition of PKC, suggesting that this enzyme may play a role in NG2/HMPG function. Direct phosphorylation of NG2/HMPG may result in a change in conformation and increased susceptibility to proteases.

PKC is thought to modulate neural cell function by regulating potassium and calcium ion channels (Nishiyama 1995).

*Insulin receptor related receptor.*

The insulin receptor (IR), insulin receptor related receptor (IRRR) and the insulin-like growth factor receptor (IGF-1R) belong to a family of tyrosine kinase receptors (Zhang 1992), and are widely distributed in mammals (Siddle 1992), where they are one of the primary regulators of metabolism and growth. (Shier 1989).

Fifteen amino acids in the third extracellular domain of NG2/HMPG (2050-2064) show 40% identity with a segment of the human IRRR. In particular three cysteine residues are conserved in NG2/HMPG, IRRR, IR and IGF-1R. In IR and IGF-1R they are thought to form intersubunit disulphide bonds, which enable the formation of the active heterotetrameric receptors (Shier 1989). NG2/HMPG may have a similar conformation.

## SUMMARY

NG2/HMPG appears to act in the transduction of signals from one side of the membrane to the other. In rat cell lines, NG2/HMPG does this by interaction with the actin cytoskeleton to influence events such as control of cell adhesion and migration. In rat neurites, NG2/HMPG employs a number of intracellular signalling molecules to inhibit cell growth, including Gi protein-mediated control of adenylate cyclase and calcium ion channel activity. NG2/HMPG in O-2A cells and smooth muscle is involved modulating the cell's response to PDGF, and as such may be important in the control of cell proliferation. The restricted expression of NG2/HMPG to developing or transformed cell types, both of which undergo high rates of cell division, supports a role for this PG in stimulation of proliferation. Structure analysis and studies using melanoma cells indicates that signal transduction involving NG2/HMPG may involve PKC and tyrosine kinase activation to bring about such effects as modulation of cell adhesion, cell spreading, proliferation and growth.

## 1.9. SUMMARY AND AIMS OF THESIS

NG2/HMPG is a structurally unique transmembrane CSPG of restricted distribution. It appears to have different roles in each of the cell types studied. It acts as a cell surface receptor for extracellular matrix molecules such as CVI and in signal transduction to regulate cellular processes such as proliferation, adhesion and spreading.

### *Developing cells and tissues*

The distribution of NG2/HMPG in rat developing tissues, and subsequent down regulation in adult cells (Nishiyama 1991a) suggests a role in early developmental stages of the NG2/HMPG-expressing tissues such as brain and cartilage.

NG2/HMPG is expressed by a unique class of rat glial cells which appear early in development, where it acts as a cell surface receptor for CVI and other ECM proteins, and binds to the actin cytoskeleton. Inability to anchor CVI correlates with production of a truncated NG2/HMPG, possibly mediated by PKC, suggesting a potential regulatory mechanism for NG2/HMPG-CVI interaction. NG2/HMPG is expressed outside the CNS in rat developing limb cartilage until the chondroblasts differentiate, at which time NG2/HMPG expression is down regulated. CVI exhibits a similar spatial arrangement to NG2/HMPG during development, suggesting a function in the growth and development of the limb cartilage and arteriole smooth muscle cells, for example mediation of cell migration, and cell-cell interactions during cell condensation or matrix organisation.

### *Transformed cells*

In human melanoma cells NG2/HMPG influences cell-ECM interaction, but not via linkage with CVI as in rat cells. It modulates cellular responses to FN via inside out regulation of  $\alpha 4 \beta 1$  integrin interaction with the substrate, possibly using the actin cytoskeleton and activation of tyrosine kinases. Interaction with ECM is important in the process of tumour metastasis, where inhibition of cell-ECM interactions is required for detachment from the primary tumour site, and migration and re-attachment to the secondary site.

### *Pathologically proliferating cells*

NG2/HMPG may also regulate cell proliferation. NG2/HMPG expression is associated with abnormally proliferating cells, for example melanoma, sarcoma, chondrosarcoma and astrocytomas. It is also associated with pericytes in the blood vessels of melanoma tumour stroma and from adult brain, and in lesions associated with vascular proliferation, for example acute myeloid and lymphoblastic leukaemia blasts and capillary endothelial cells of glioblastoma tumour types. *In vitro* rat postnatal smooth muscle and O-2A progenitor cells require NG2/HMPG and PDGF $\alpha$ -R for optimal response to PDGF AA. All NG2/HMPG-positive cell types express the PDGF $\alpha$ -R and respond to PDGF by cell division and chemotaxis. They also retain the ability to divide throughout the life of the organism. Despite differences in morphology, vascular smooth muscle cells, brain endothelial cells and O2A progenitor cells can still divide after the appropriate stimulus. Injury to tissue provides such as stimulus, which promotes cell division of NG2/HMPG expressing cells. NG2/HMPG is therefore a marker for cells that retain stem cell-like state, or become hyperproliferative. Expression in developing cells, transformed cells, and cells associated with other proliferative disorders suggests NG2/HMPG may have a role in upregulating or maintaining proliferation of cells, perhaps by regulation of the cell response to PDGF.

### *Adult Cells and Tissues*

Mature cell types also express NG2/HMPG, for example smooth muscle cells in the rat aorta. The expression of NG2/HMPG in mature CNS is upregulated after injury. NG2/HMPG has been shown to inhibit neurite elongation stimulated by L1 and LN *in vitro*, suggesting a role in the guidance of growing axons and repair of damaged tissue. The function of NG2/HMPG is unknown, but it may act to regenerate tissues after injury or disease by regulating cell re-growth as it does during development.



### *Expression of NG2/HMPG as two forms.*

NG2/HMPG is expressed as two forms at the cell surface, which raises questions as to which is functionally active. The core protein is responsible for interaction with ECM proteins, including CVI and cell surface receptors in neurites (Burg 1996, Dou 1997). However, the CS GAGs are essential for the correct folding of NG2/HMPG that allows this direct interaction. Melanoma cell interaction with FN also requires CS chains (Iida 1992). Purified CS interacts with FN, suggesting that CS GAGs are responsible for mediating CSPG adhesion to this protein (Barkalow 1994). CS GAGs play a role in neural development (Sorrelli 1996), PGs such as aggrecan fail to inhibit neurite growth after removal of CS chains (Grumet 1996). CD44 and  $\beta$ -glycan exist at the cell surface as core proteins and PG forms (Jalkanen 1988, Lopez-Casillas 1991). CD44 mediates cell adhesion to a number of ECM proteins, including FN. The CS rich form of CD44 interacts with FN, whereas the core protein is incapable of binding to FN, suggesting that the CS chains mediate the interaction (Lesley 1993).

Expression as both a CSPG and protein core may regulate the activity of NG2/HMPG at the cell surface, however the amounts and functions of the two different forms in different tissues has not been characterised.

### *Aims and objectives*

Preliminary investigation reveals a role for NG2/HMPG in cell adhesion with ECM molecules. Several ECM proteins, including CVI, have been shown to bind NG2/HMPG purified from rat cell lines (Burg 1996). Rat chondrocytes produce CVI, which shows a similar pattern of expression to NG2/HMPG *in vivo*. This suggests that NG2/HMPG and CVI interactions may have a functional relevance in cartilage in the developing rat (Stallcup 1990). NG2/HMPG is a receptor for CVI for rat neural cells, therefore it is likely that it may act as a rat chondrocyte CVI receptor. Similarly, if expressed in human cartilage NG2/HMPG may act as a CVI receptor. Human cartilage expresses CVI in the pericellular matrix, which is thought to function as a link between the ECM and the cell surface, maintaining the structure of

the tissue, and transmitting signals between the inside and outside of the cell (Poole 1992). If NG2/HMPG is expressed in human cartilage it may mediate interactions with CVI and play an important role in cell adhesion and regulation of chondrocyte function, either during development or in the adult cell. NG2/HMPG may be important in cell-ECM interactions in either normal or OA cartilage. Alternatively, NG2/HMPG may be involved in cell adhesion to CII or FN. NG2/HMPG has previously been shown to interact directly with CII and to modulate human melanoma cell interaction with FN (Burg 1997, Iida 1995). FN is localised to the pericellular matrix of human cartilage, and CII is the major collagen component of the entire cartilage ECM (Clemmensen 1982).

The purpose of this project was to determine whether NG2/HMPG is expressed by human fetal and adult articular chondrocytes and, if so, to determine its biochemical structure and possible cell function. This study also aimed to compare expression in adult normal and OA cartilage, and to investigate a potential role in the disease process.



## 2.0. MATERIALS AND METHODS.

All reagents were purchased from Sigma, Poole UK, unless otherwise stated.

Antibodies, solutions and tissues sources used in this study are listed in appendices I, II and III.

All work with human material and cells was carried out in a class I tissue culture hood. Gloves, lab coat and oversleeves were worn when handling tissue. If tissue was found to be infected the joint was abandoned and the hood fumigated.

Fetal knee joints were obtained from second trimester miscarriages. Normal and osteoarthritic adult articular cartilage was obtained at post mortem from patellae, tibial plateaux and femoral condyles of adult knee joints.

### 2.1. ASSESSMENT OF CARTILAGE FOR OSTEOARTHRITIS

Joints were assessed for the presence or absence of osteoarthritis macroscopically and graded according to Collins (Collins 1960) (**TABLE 2.1.**) (**PLATES 2.1a-f.**)

**TABLE 2.1.**

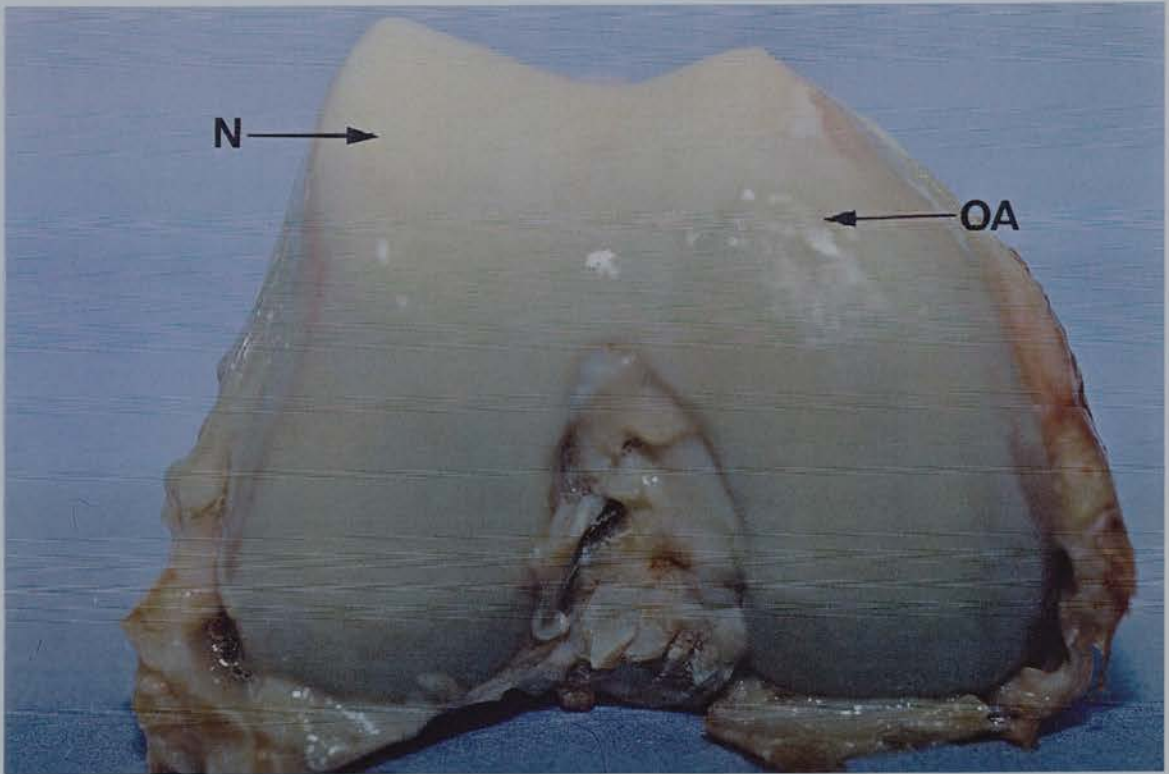
Collins macroscopic grading of cartilage for disease (Collins 1960)

GRADE	DESCRIPTION
0	normal healthy joint with smooth cartilage
1	superficial flaking of cartilage in areas of pressure and movement
2	more extensive destruction of cartilage not denuding bone
3	total loss of cartilage in one or more pressure areas and obvious marginal osteophytes
4	complete loss of cartilage from large areas with eburnation of bone, prominent osteophytes



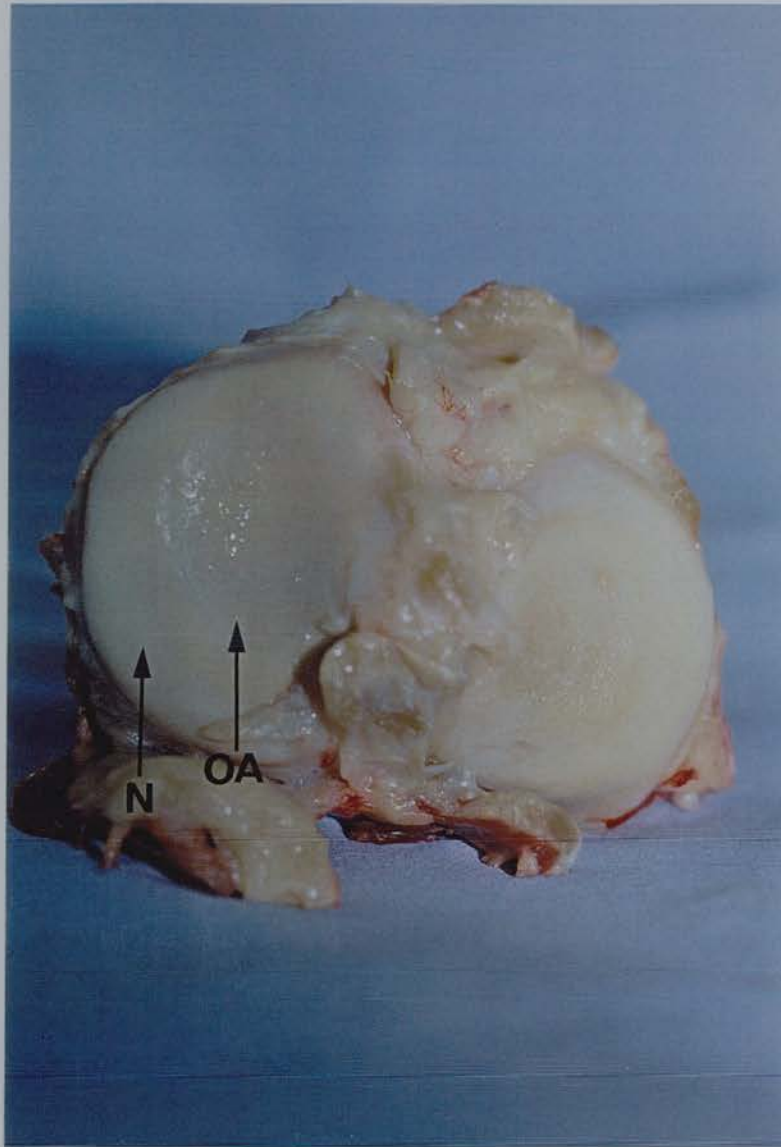
**PLATE 2.1a.**

Patella obtained from knee joint of 68 year old female at post mortem, showing Grade 1 OA cartilage (OA) with superficial flaking



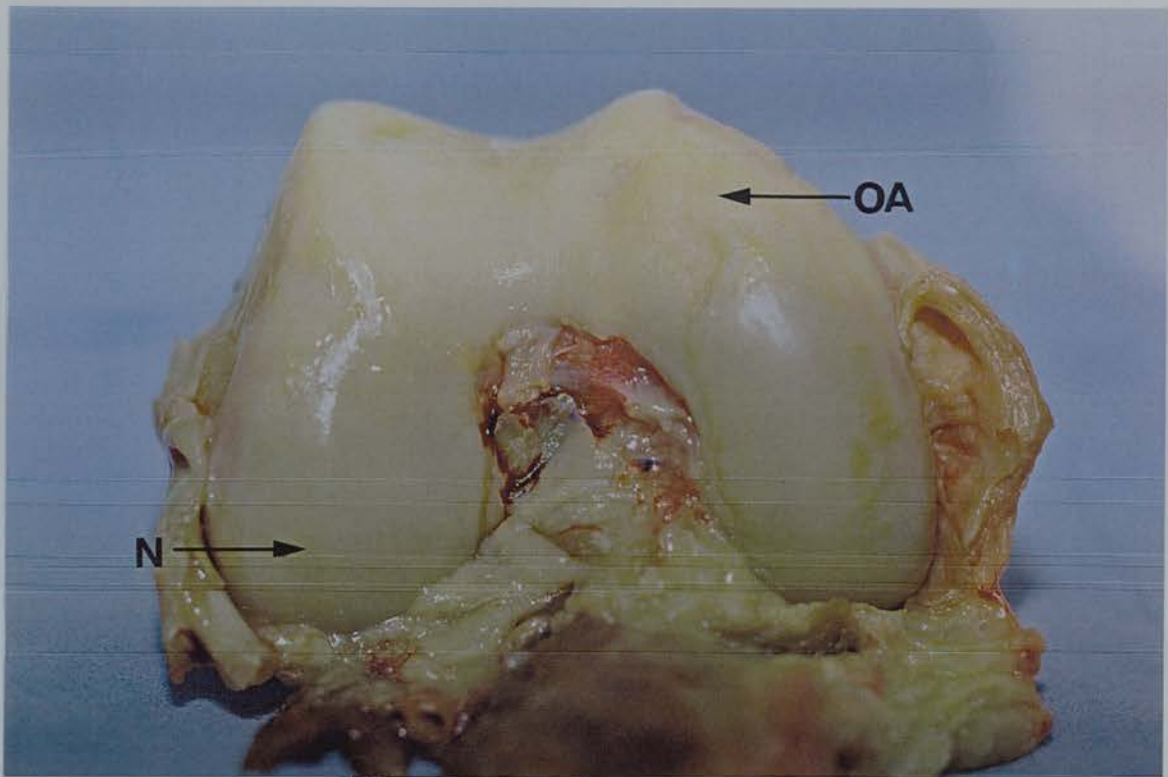
**PLATE 2.1b.**

Femoral condyle obtained from knee joint of 68 year old female at post mortem, showing areas of normal smooth cartilage (N) with no destruction and Grade 1 OA cartilage (OA) with superficial flaking.



**PLATE 2.1c.**

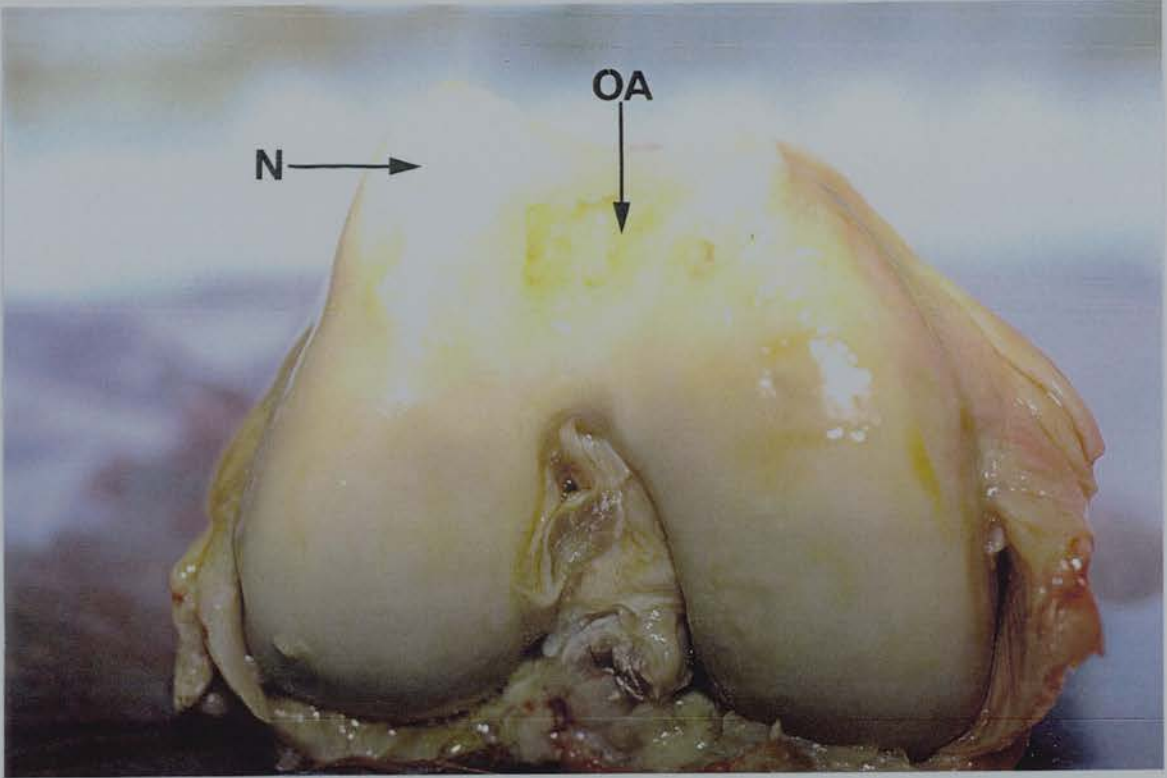
Tibial plateau obtained from knee joint of 68 year old female at post mortem with areas of normal smooth cartilage (N) and Grade 2 OA cartilage (OA), exhibiting destruction of superficial and deep layers.



**PLATE 2.1d.**

Femoral condyle obtained from knee joint of 72 year old female at post mortem with areas of normal smooth cartilage (N) and Grade 2 OA cartilage (OA), exhibiting destruction of superficial and deep layers.





**PLATE 2.1e.**

Femoral condyle obtained from knee joint of 58 year old male at post mortem with areas of normal smooth cartilage (N) and Grade 3 OA cartilage (OA), with extensive destruction of deep layer cartilage and exposure of bone in areas of pressure.



**PLATE 2.1f.**

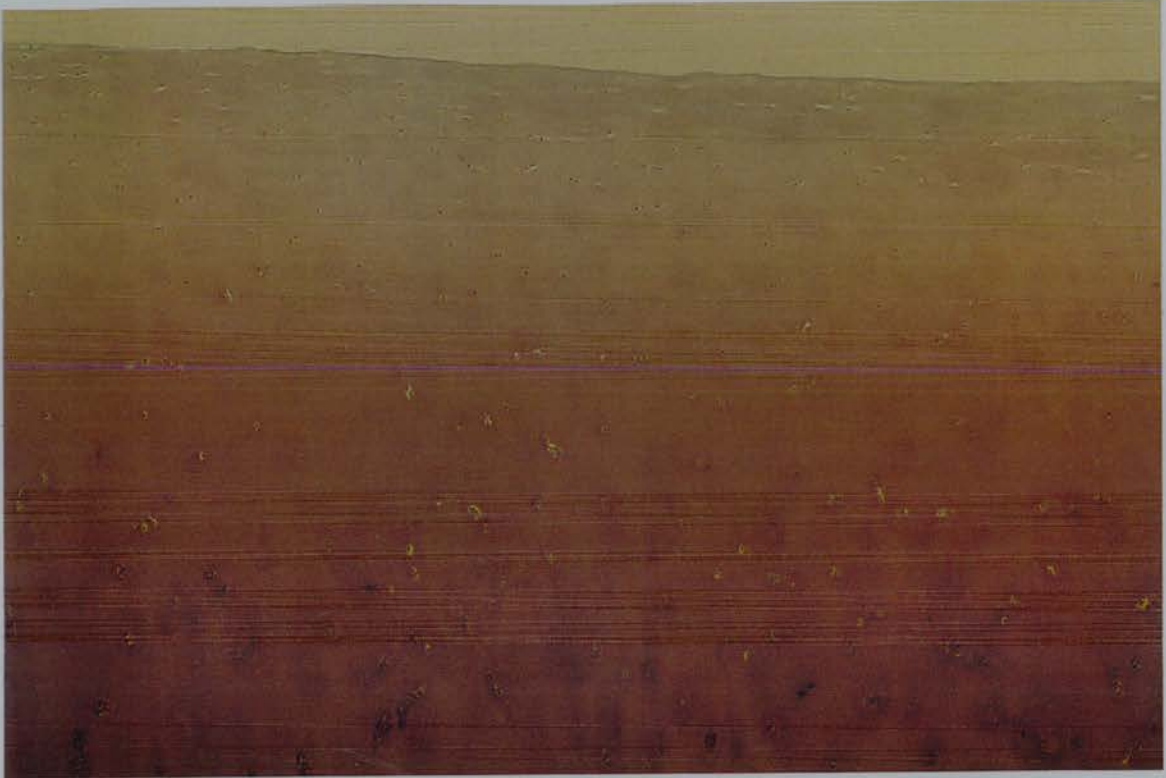
Femoral condyle obtained from knee joint of 83 year old male at post mortem, with areas of Grade 4 OA cartilage (OA), with complete loss of cartilage from large areas of the joint and osteophyte formation (OP).

Cartilage was also assessed microscopically following safranin-O staining (Mankin 1971) according to **TABLE 2.2 (PLATES 2.2a, b.)**. Tissue samples were snap frozen in liquid nitrogen and 4 µm sections cut with a Bright cryostat. Sections were mounted on PLL coated glass slides and fixed with acetone (Fisher Scientific, Loughborough UK) for 20 min at -20 °C. Sections were stained with haematoxylin blue (Fisons, USA) for 10 min, washed with water, stained for 3 min with fast green diluted 1:5000 in water, washed in 1 % acetic acid, then stained with 0.1 % safranin-O for 5 min. Sections were then dehydrated in ascending grades of alcohol (64 %, 74 %, absolute), cleared in xylene (Genta Medical, UK) and mounted in pertex (Cellpath, UK).

**TABLE 2.2.** Microscopic grading of cartilage for disease (Ostergaard 1997b).

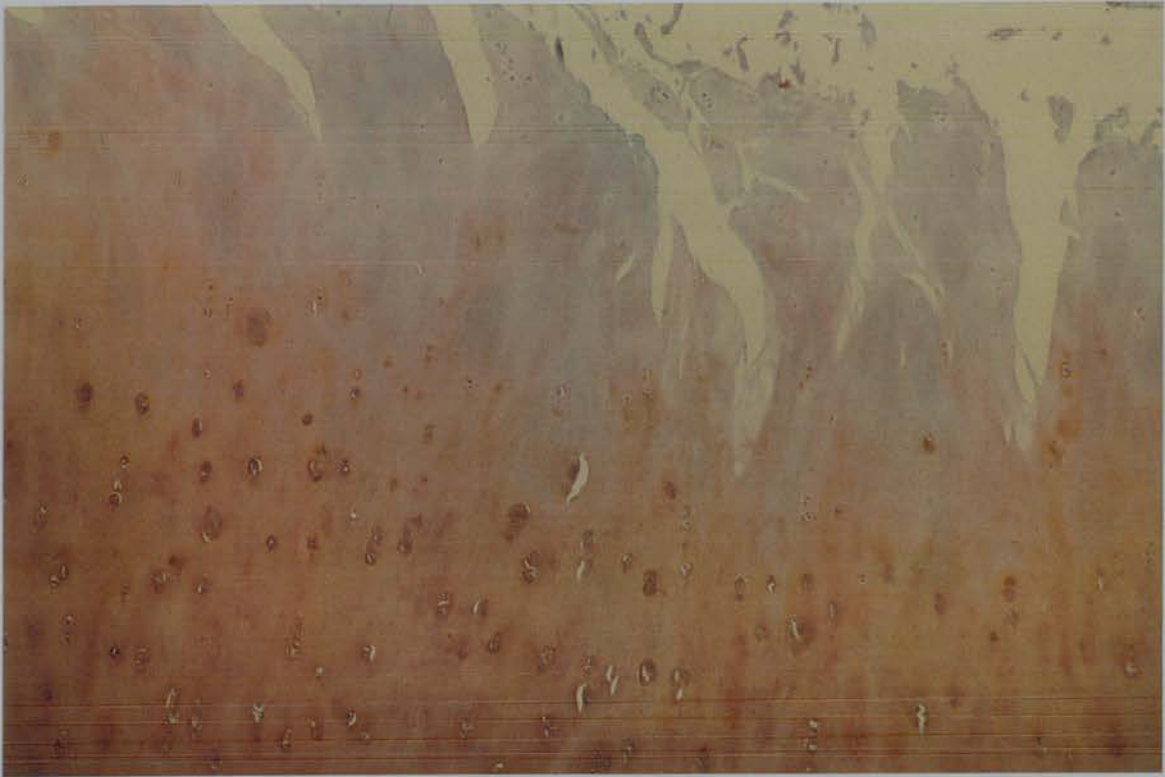
CATEGORY	SUBCATEGORY	SCORE
Structure	normal	0
	surface irregularities	1
	pannus + surface irregularities	2
	clefts to transitional zone	3
	clefts to radial zone	4
	clefts to calcified zone	5
	complete disorganisation	6
Cells	normal	0
	diffuse hypercellularity	1
	cloning	2
	hypocellularity	3
Safranin-O staining	normal	0
	slight reduction	1
	moderate reduction	2
	severe reduction	3
	no dye noted	4
Total /13		





**PLATE 2.2a.**

Section of normal cartilage from knee joint of 53 year old male, stained with safranin-O used to assess the degree of disease. Original magnification x40. Normal cartilage exhibits strong staining of proteoglycans with safranin-O, no disruption of tissue structure and no cell clustering or cloning.

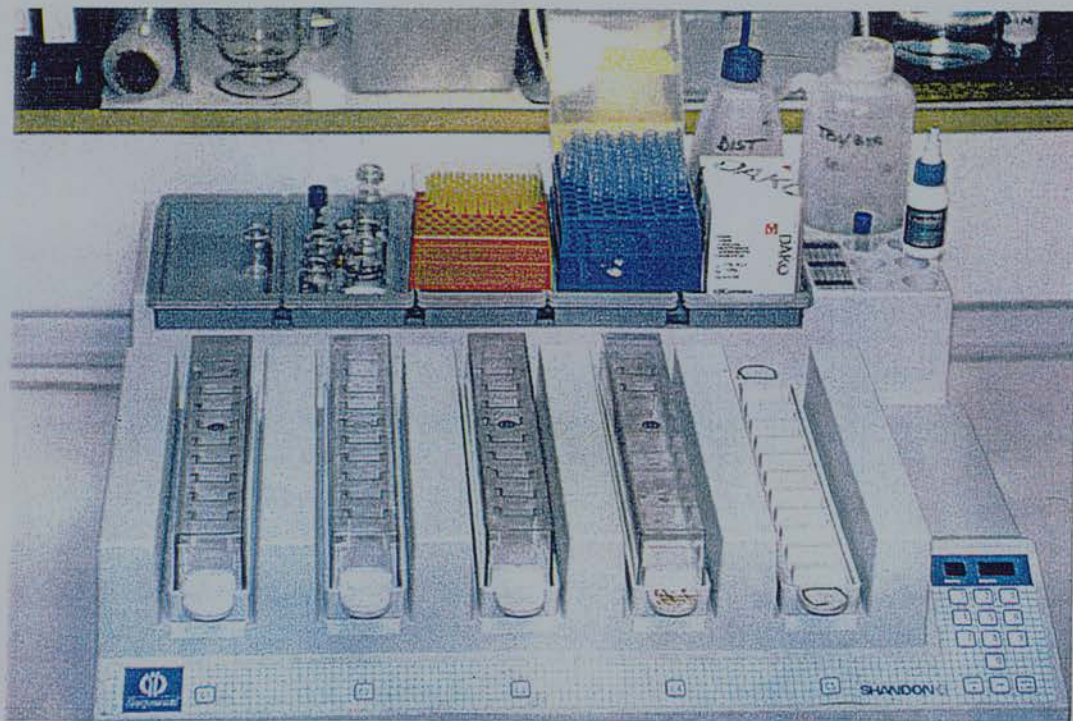


**PLATE 2.2b.**

Section of OA cartilage from knee joint of 89 year old female, stained with safranin-O used to assess the degree of disease. Original magnification x40. The OA section exhibits a reduction uptake of safranin-O, indicating a decrease in proteoglycan synthesis, surface irregularities and clefts, hypercellularity and cell cluster formation.

## 2.2. IMMUNOHISTOCHEMISTRY

Tissue samples were snap frozen in liquid nitrogen and 4 µm sections cut with a Bright cryostat. Sections were mounted on PLL coated glass slides, placed into slide racks and fixed with acetone for 20 min at -20 °C. Sections were placed in Tris buffered saline (TBS) pH 7.6 for 5 min before loading into a Shandon Sequenza (PLATE 2.3.) Sections were incubated in 100 µl normal rabbit serum (Law hospital, UK) diluted 1:5 in TBS for 10 min, then 100 µl primary antibody diluted optimally in normal serum/TBS for 30 min. Sections were stained with antibodies against rat NG2 (N143.8) and HMPG (M28B5, 9.2.27). Positive controls were obtained by staining with KZ1 (anti-CD44) and negative controls by omission of the primary antibody. Sections were washed twice for 5 min in TBS. Biotinylated rabbit anti-mouse secondary antibody diluted optimally in 100 µl normal serum/TBS was applied for 30 min. At this stage the avidin-biotin (AB) conjugated horse radish peroxidase (HRP) complex (DAKO, UK) was prepared by adding one drop each of solutions A and B to 5 ml AB buffer. Sections washed twice for 5 min in TBS, then incubated in AB-HRP complex for 30 min. Sections were washed twice for 5 min in TBS then staining was visualised using 100 µl diaminobenzidine (DAB) substrate solution for 3 min. Sections were removed from the sequenza and washed in running tap water. Sections were counter stained lightly in haematoxylin blue for 30 s, blued up by dipping in Scott's tap water substitute for 30 s and dehydrated in ascending grades of alcohol (64 %, 74 %, absolute). Sections were cleared by incubation in xylene for 5 min and mounted in pertex mountant (Cellpath, UK). Sections were examined using Olympus B071 light microscope.



**PLATE 2.3.**

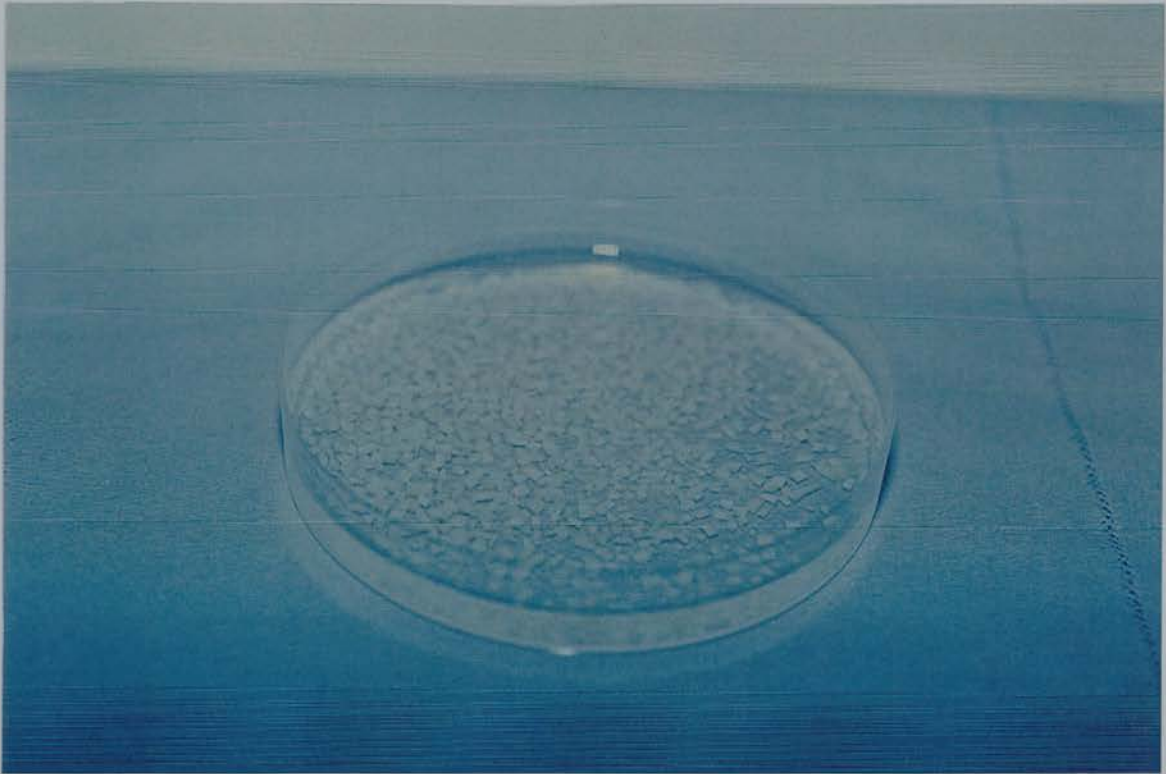
The Shandon sequenza used for immunohistochemical staining of cartilage sections.



## **2.3. WESTERN BLOTTING**

### **2.3.1. Isolation and culture of chondrocytes.**

Knee joints were obtained at autopsy. Articular cartilage was aseptically removed from macroscopically normal or osteoarthritic appearing patellae, tibial plateaux and femoral condyles of knee joints and graded macro and microscopically. Cartilage was cut into small pieces and incubated in antimicrobial solution for 1 h (**PLATE 2.4**). Cartilage pieces were washed twice with phosphate buffered saline (PBS) (Oxoid, Basingstoke UK) and sequentially digested for 30 min at 37 °C with 0.25 % trypsin (GIBCO, Paisley UK), then for 24 h at 37 °C with 3 mg/ml collagenase. The sample was checked for digestion by examination under the microscope. Single cells should be clearly visible. If it was possible to see extracellular matrix around the cells and/or the cells were still clumped together, the collagenase digestion was repeated. The cell suspension was collected using a sterile pastette and strained through a sterile strainer to remove undigested cartilage fragments. The cell suspension was centrifuged at 1000 rpm, the supernatant discarded and the pellet re-suspended in PBS. This was repeated twice more to give three PBS washes. The cells were re-suspended in 10 ml serum-free HAMs F12 medium (GIBCO, Paisley UK) and filtered through a 70 micron cell sieve (Falcon, Becton Dickinson UK) to remove large cell clumps and pieces of debris. Cells were counted using a haemocytometer and viability checked using trypan blue. Cell suspension was made up to give  $1 \times 10^5$  cells/ml in HAMs F12 containing 10 % FCS (Advanced Protein Products, West Midlands UK). Large culture flasks (Nunc, Life Technologies UK) were seeded with 70 ml of cell suspension, medium flasks (Nunc, Life Technologies UK) with 30 ml to give a cell density of  $4 \times 10^4$  cells/cm<sup>2</sup>. Cells were grown for 5-10 days in monolayer culture at 37 °C in HAMs F12 medium supplemented by 10 % FCS, 1 % penicillin, streptomycin and fungizone and the medium was changed every 3-4 days.



**PLATE 2.4.**

Cartilage after removal from the tibial plateau and dissection. Cartilage was obtained from a normal knee joint from a 77 year old male.

### **2.3.2. Assessment of cell number and viability**

A haemocytometer was washed in 74 % ethanol, rinsed under tap water and dried. With cover slip in place 10 µl cell suspension was added to one chamber by touching the tip of the pipette to the edge of the coverslip and allowing the chamber to fill by capillary action. To the other chamber 10 µl of a 1:1 cell suspension:trypan blue was added in the same way. The number of cells without trypan blue was assessed by counting all the cells in the 1 mm centre square and four 1 mm corner squares. Each square represents a total volume of  $10^{-4}$  cm<sup>3</sup>, and since 1 cm<sup>3</sup> is equal to 1 ml the number of cells per ml is equal to the average count per square x dilution factor x  $10^4$ . The total number of cells is equal to the number of cells per ml x the original volume of fluid from which the cell sample was removed. Viability of cells was assessed within 2 min of trypan blue addition. Cell viability (%) is equal to the total number of viable cells (unstained with trypan blue) divided by the total number of cells (stained and unstained) x100.

### **2.3.3. Removal of cell monolayers from tissue culture flasks**

10 ml of 0.02 % EDTA in PBS per flask was warmed to 37 °C. The cell monolayers were washed twice with PBS and then incubated with the warm EDTA/PBS for 10 min at 37°C. The flask was tapped to release cells and detachment or rounding up was looked for using a light microscope. The cell suspension was decanted into sterile universals, the flask rinsed with PBS and this wash added to the rest. The cell suspensions were centrifuged at 1000 rpm for 10 min at 10 °C. The supernatant was discarded and the cell pellet re-suspended in PBS. The centrifugation was repeated twice, combining pellets from the same preparation into one universal. The final cell pellet was re-suspended in a volume of medium appropriate for the experiment and filtered through a 70 micron cell sieve to remove clumps. The cell number and viability was determined to be greater than 95 % by trypan blue exclusion.

#### **2.3.4. Chondroitinase digestion**

Cells were incubated with 1 U/ml chondroitinase ABC for 1 h at 37 °C before protein extraction.

#### **2.3.5. Cell lysis**

Cells were centrifuged at 1000 rpm for 10 min at 10 °C. The supernatant was discarded and the cell pellet re-suspended in 1 ml lysis buffer. The cells were allowed to lyse on ice or at 4 °C for 30 min and then microcentrifuged at high speed for 15 min at 4 °C. The supernatant was kept and either used immediately or frozen at -20 °C for future use. The pellet containing nuclear debris was discarded.

#### **2.3.6. Lowry determination of protein concentration**

Bovine serum albumen (BSA) (First link, UK) standards increasing in concentration from 0 (S1) - 400 (S8) µg/ml were set up in triplicate and vortexed. Test sample (T) tubes were set up in triplicate by adding 5 µl sample to 195 µl 0.1 N NaOH. Blank tubes (B) containing 200 µl NaOH were also set up in triplicate. The tubes were vortexed to mix the sample. To all tubes 1 ml of alkaline carbonate solution was added, the tubes were vortexed and allowed to stand at room temperature for 10 min. To each tube 100 µl Folin's reagent was added, the tubes were vortexed and allowed to stand at room temperature for 30 min. An aliquot of 200 µl of each sample was transferred to a 96 well tissue culture plate (Nunc, Life Technologies UK) according to the template in **TABLE 2.3.**, and the absorbance read on a Dynatech MR 500 microplate reader at 570 nm. The average concentration for each sample was calculated using a standard curve of BSA protein concentrations (**GRAPH 2.1.**).



**TABLE 2.3.**

Template used for Lowry determination of protein concentration

	1	2	3	4	5	6	7	8	9	10
A	B1	S1.1	S1.2	S1.3	T1.1	T1.2	T1.3	T9.1	T9.2	T9.3
B	B2	S2.1	S2.2	S2.3	T2.1	T2.2	T2.3	T10.1	T10.2	T10.3
C	B3	S3.1	S3.2	S3.3	T3.1	T3.2	T3.3	T11.1	T11.2	T11.3
D	B4	S4.1	S4.2	S4.3	T4.1	T4.2	T4.3	T12.1	T12.2	T12.3
E	B5	S5.1	S5.2	S5.3	T5.1	T5.2	T5.3	T13.1	T13.2	T13.3
F	B6	S6.1	S6.2	S6.3	T6.1	T6.2	T6.3	T14.1	T14.2	T14.3
G	B7	S7.1	S7.2	S7.3	T7.1	T7.2	T7.3	T15.1	T15.2	T15.3
H	B8	S8.1	S8.2	S8.3	T8.1	T8.2	T8.3	T16.1	T16.2	T16.3

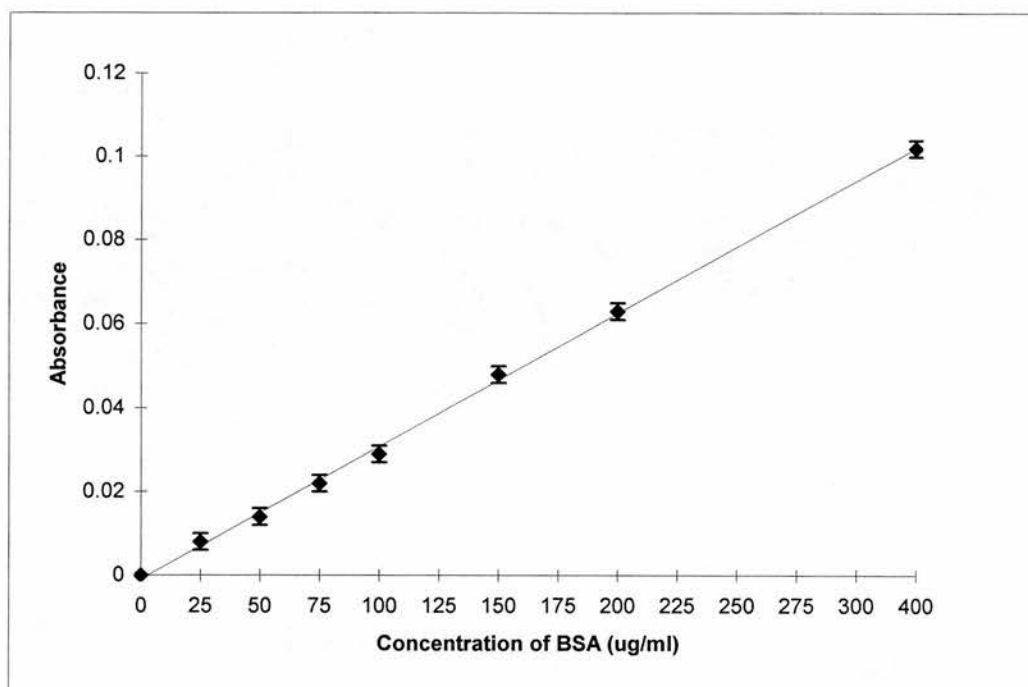
B blank wells containing NaOH

S standard wells containing BSA increasing in concentration from 0-400 µg/ml

T test wells containing protein samples

## GRAPH 2.1

BSA standard protein concentration.



The values shown represent the mean of 3 experiments  $\pm$  SEM.

### **2.3.7. Sodium Dodecyl Sulphate Poly Acrylamide Gel**

#### **Electrophoresis (SDS-PAGE)**

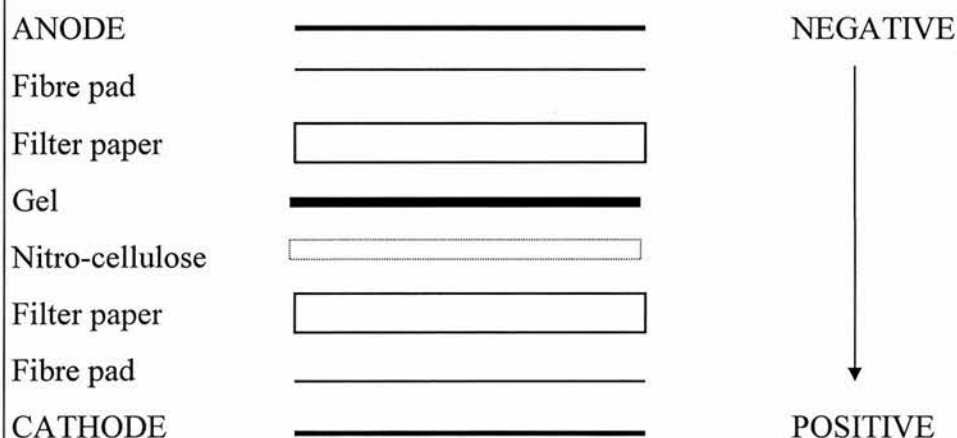
Proteins were analysed by SDS-PAGE according to the method of Laemmli (Laemmli 1970), using equipment from Bio-Rad. Glass gel plates (12 cm x 10 cm and 12 cm x 9 cm ) and spacers were cleaned with ethanol. Glass plate sandwiches were assembled into the clamp assembly and the unit transferred to the casting stand. A comb was placed in between the plates and a mark made 1 cm below the edge of the teeth. A separating gel solution was prepared of appropriate acrylamide (National diagnostics, UK) percentage for the resolution required. The solution was mixed and poured between the plates up to the mark using a pasteur pipette. The solution was overlaid with distilled water and allowed to polymerise for 45 min. The water was poured off and the gel dried with filter paper. The stacking gel solution was prepared and poured over the separating gel to the top of the plates. A 10 or 5 well comb was placed in the sandwich and the gel allowed to polymerise for 30 min. When polymerised the comb was removed, the clamp assemblies removed from the casting stand and attached to the inner cooling core. The electrode buffer was prepared from a 10x stock by diluting 50 ml with 450 ml dH<sub>2</sub>O. The top buffer chamber was filled with electrode buffer. Samples were prepared by adding 1 part sample to 3 parts sample buffer and boiled for 3 min and then loaded onto the gel. The inner cooling core was placed into the cell and electrode buffer poured into the lower chamber so that at least 1 cm of gel was covered. The samples were electrophoresed through the gel at a constant voltage of 200 V and stopped when the line of bromophenol blue dye reached the bottom of the resolving gel. SDS-PAGE was carried out using a 5 % acrylamide separating gel with a 4.5 % acrylamide stacking gel and a 3-20 % acrylamide gradient separating gel with a 2.5 % acrylamide stacking gel.

### 2.3.8. Electrophoretic transfer of proteins to nitro-cellulose

The gel assembly was removed from the gel apparatus, the glass plates separated and the gel removed. For each gel a piece of nitro-cellulose (Amersham, UK) and two pieces of 3MM filter paper (Whatman, UK) was cut to a size 0.5 cm bigger than the gel. These, along with two fibre pads per gel were equilibrated in transfer buffer for 10 min. The plastic holder for the gel membrane sandwich was opened, a nylon pad placed onto it followed by a piece of filter paper, then the gel, the nitro-cellulose, the second piece of filter paper and finally the second fibre pad. The holder was closed and squeezed to exclude air bubbles then placed in the blotting apparatus ensuring that the nitro-cellulose lies between the cathode and the gel (**FIGURE 2.1.**). The tank was filled with transfer buffer sufficient to cover the gel/membrane sandwich without touching the electrodes and transfer was carried out at 100V for 1 h, using a pre-frozen cooling unit.

**FIGURE 2.1.**

Assembly of blotting apparatus



### **2.3.9. Development of blot using alkaline phosphatase**

The plastic holder was removed from the blotting apparatus and opened on a flat surface. The nitro-cellulose was removed and marked for orientation by cutting off the top left hand corner. The nitro-cellulose was placed in 20 ml blocking solution and incubated for 1 h at room temp or overnight at 4 °C. The blocking solution was poured off and the membrane was washed 3 times with 20 ml TBS Tween (TBST) with rotation. The membrane was incubated with primary anti-HMPG antibodies M28B5 or 9.2.27, anti-FN antibody IST-4 or anti-phosphothreonine antibody diluted optimally in 20 ml TBST for 1 h with rotation or over night at 4°C, then washed as above. The membrane was incubated with secondary rabbit anti mouse biotinylated antibody diluted 1:1000 in 20 ml TBST for 1 h with rotation, then washed as above. The membrane was then incubated in AB-Alkaline phosphatase (AP) complex (DAKO, UK) in TBST for 1 h with rotation and then washed as above. The membrane was then incubated in AP substrate solution for 5-20 min in the dark with rotation. The membrane was washed in distilled water.

### **2.3.10. Development of blot using Enhanced Chemiluminescence (ECL)**

The plastic holder was removed from the blotting apparatus and opened on a flat surface. The nitro-cellulose was removed and marked for orientation by cutting off the top left hand corner. The nitro-cellulose was placed in 20 ml blocking solution (Amersham ECL kit) and incubated for 1 h at room temperature or overnight at 4 °C. The blocking solution was poured off and the membrane was washed 3 times with 20 ml TBST with rotation. The membrane was incubated with primary antibody diluted optimally in 20 ml TBST for 1 h with rotation or overnight at 4 °C, then washed for 5 min 3 times. The membrane was incubated with secondary rabbit anti mouse biotinylated antibody diluted 1:1000 in 20 ml TBST for 1 h with rotation, then washed as above. The membrane was incubated in AB-HRP complex in TBST for 1 h at room temp with rotation. The membrane was washed once for 15 min with TBST and twice for 5 min with TBST with rotation. The proteins were then

visualised by ECL detection. Onto one membrane 5 ml of solution A and 5 ml of solution B (Amersham, UK) were poured and the membrane shaken gently for 1 min. The membrane was removed, the fluid drained off and the membrane wrapped in Saran wrap. Any air pockets were smoothed out. The blot was placed in an autoradiography cassette and exposed to ECL hyper film (Amersham, UK) for 1 min. The film was developed by hyperprocessor (Amersham, UK) and the blot re-exposed as required depending on the strength of the signal.

### 2.3.11. Estimation of protein size

The molecular weight of proteins analysed by SDS-PAGE was determined by comparison with proteins of known size electrophoresed on the same gel. The proteins used for these standards are given in **TABLE 2.4.** (Novex, Oxon UK).

**TABLE 2.4.**

Protein standards and corresponding molecular weights used to estimate size of sample protein.

PROTEIN	MOLECULAR WEIGHT (kD)
Myosin	250
Phosphorylase B	148
Glutamic dehydrogenase	60
Carbonic anhydrase	42
Myoglobin-blue	30
Myoglobin-red	22
Lysozyme	17
Aprotinin	6
Insulin	4

### **2.3.12. Quantification of band intensity**

Equal amounts of protein, determined by Lowry assay, were loaded onto gels. Band intensity was compared by densitometric analysis using Enhanced Analysis System (EASY) computer and hardware (Herolab, Scotlab UK). A value of 100 was assigned to the most intense band and the intensity of other bands expressed relative to this.

### **2.3.13. Statistical analysis**

Non parametric Mann Whitney U tests were used to determine the significance of results. This test avoids assumptions about the underlying sample distribution and allows comparison of data from two different populations with numerically graded data. Significant results correspond to p values less than 0.05.



## 2.4. IMMUNOPRECIPITATION

Immunoprecipitation of NG2/HMPG was carried out as previously described (Stallcup 1990). Chondrocytes were isolated from knee joints as described above and cultured for 5-10 days. Cells were removed from culture flasks using PBS containing 0.02 % EDTA and washed twice with PBS. Cells were counted and viability determined by trypan blue exclusion. Cells in 1 ml PBS were labelled with  $I^{125}$  by incubation with 5  $\mu$ M glucose, 24 mU glucose oxidase, 20 mU lactoperoxidase and 200  $\mu$ Ci  $NaI^{125}$  (BDH, Lutterworth UK) at room temperature with occasional mixing. After 20 min the reaction was stopped by the addition of 2 mM KI. The labelled cells were washed with PBS and 1 mM KI and lysed as above. Aliquots of the extract were incubated for 2 h on ice with the primary mouse monoclonal antibody M28B5 or 9.2.27 and immunoprecipitation accomplished by subsequent incubation with protein A-Sepharose (Pharmacia, St. Albans UK) for 2 h at 4 °C. The immune complexes were washed three times with PBS containing 0.2 % NP-40 and 0.02 % SDS, then dissolved in electrophoresis sample buffer. SDS-PAGE was performed using gels with a stacking gel of 2.5 % acrylamide and a separating gel with a gradient of 3-20 % acrylamide, dried with Dry Ease gel drying system (Novex, Oxon UK) and used for autoradiography with Kodak film (Kodak, UK). Negative controls were obtained by omission of primary antibody and labelling efficiency was assessed by omission of protein A-Sepharose.

## **2.5. IMMUNOFLUORESCENCE**

### **2.5.1. Single immunofluorescent staining of cultured chondrocytes**

Chondrocytes from normal and OA adult articular cartilage were cultured at a concentration of  $5 \times 10^4$ /ml as a monolayer for 5-10 days on 53 mm tissue culture grade petri dishes (Nunc, Life Technologies UK). Non specific background was blocked by incubation with normal rabbit serum diluted 1:10 in 1 ml TBS for 1 h. The serum was tapped off and the cells were incubated for 30 min with primary antibody diluted optimally in 1 ml HAMS F12 medium with 2 % FCS. Cells were stained with monoclonal antibodies against HMPG (M28B5 and 9.2.27). The negative control was obtained by omission of primary antibody. The cells were washed with 2 ml HAMS F12 medium with 2 % FCS three times for 5 min with rotation. The cells were incubated with rabbit anti-mouse fluorescein isothiocyanate (FITC) labelled secondary antibody diluted 1:100 in 1 ml HAMS F12 medium with 2 % FCS for 30 min in the dark. The cells were washed with 2 ml PBS three times for 5 min with rotation. The cells were then fixed using 1 ml of a 1:1 methanol/acetone mixture per dish for 5 min at  $-20^\circ\text{C}$ . Coverslips were cleaned with ethanol and allowed to air dry, then the cells were mounted using Citifluor mountant (UKC Chem. lab., UK) and the edges of the coverslip secured by painting with clear nail varnish. The cells were visualised using a Zeiss axiophot fluorescence microscope.

### **2.5.2. Double immunofluorescent staining of cultured chondrocytes using mono and polyclonal antisera.**

Chondrocytes from normal adult articular cartilage were cultured at a concentration of  $5 \times 10^4$ /ml as a monolayer for 5-10 days on 53 mm tissue culture grade petri dishes. The cells were incubated for 1 hour with normal goat serum diluted 1:10 in TBS, then simultaneously with primary mouse monoclonal anti-HMPG M28B5 and rabbit polyclonal PDGF $\alpha$ -R, FN, CII or CVI antibodies diluted optimally in 1 ml HAMS F12 medium with 2 % FCS, for 30 min RT. The cells were washed with 2 ml HAMS F12 medium with 2 % FCS three times for 5 min with rotation. The cells were incubated simultaneously with appropriately labelled secondary antibodies (goat anti

mouse tetramethyl rhodamine thiocyanate (FITC) conjugate and goat anti rabbit TRITC conjugate for FN, CII and CVI, and goat anti mouse TRITC conjugate and goat anti rabbit FITC conjugate for PDGF $\alpha$ -R) diluted 1:100 in 1 ml HAMS F12 medium with 2 % FCS for 30 min in the dark. The cells were washed with 2 ml PBS three times for 5 min with rotation. The cells were then fixed using 1 ml of a 1:1 methanol/acetone mixture per dish for 5 min at -20 °C. Coverslips were cleaned with ethanol and allowed to air dry, then the cells were mounted using Citifluor mountant and the edges of the coverslip secured by painting with clear nail varnish. The cells were visualised using a Zeiss axiophot fluorescence microscope.

### **2.5.3. Double immunofluorescent staining of cultured chondrocytes using rhodamine phalloidin and monoclonal antisera**

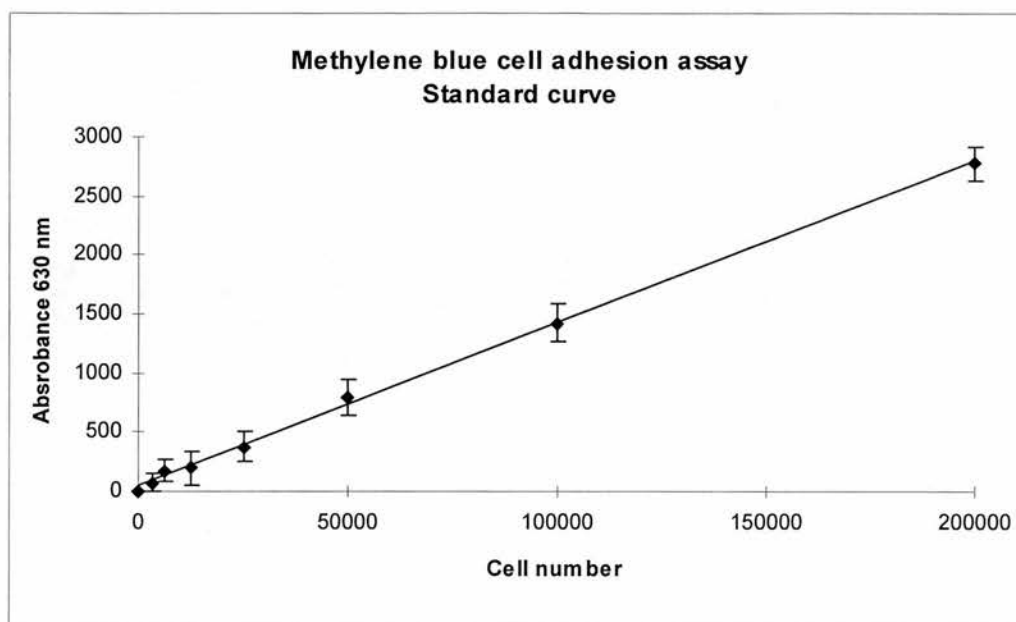
Chondrocytes from normal adult articular cartilage were cultured at a concentration of  $5 \times 10^4$ /ml as a monolayer for 5-10 days on 53mm tissue culture grade petri dishes. The cells were then fixed using 1 ml of a 1:1 methanol/acetone mixture per dish for 5 min at -20 °C. The cells were permeabilised in 1 ml 0.1 % Triton x-100 in TBS for 5 min. The cells were washed using 2 ml TBS with rotation three times for 5 min. The cells were treated with rhodamine conjugated phalloidin diluted 1:20-50 with 1 ml TBS for 30 min. This and all subsequent steps were carried out in the dark. The cells were washed using 2 ml TBS at room temp with rotation three times for 5 min. Non specific background was blocked by incubation with normal rabbit serum diluted 1:10 in 1 ml TBS for 1 h. The serum was tapped off and the cells were incubated overnight at 4 °C with primary anti-HMPG monoclonal antibody M28B5 diluted optimally in 1 ml TBS. The cells were washed with 2 ml TBS three times for 5 min with rotation. The cells were incubated with rabbit anti-mouse FITC labelled secondary antibody diluted 1:100 in 1 ml TBS for 30 min in the dark. The cells were washed with 2 ml TBS three times for 5 min with rotation. Coverslips were cleaned with ethanol and allowed to air dry, then the cells were mounted using Citifluor mountant and the edges of the coverslip secured by painting with clear nail varnish. The cells were visualised using a Zeiss axiophot fluorescence microscope.

## 2.6. CELL ADHESION

### 2.6.1. Methylene blue cell adhesion assay.

96 well tissue culture flat bottomed plates were coated with 10 µg/ml of ECM proteins FN, CII and CVI in 50 µl PBS, overnight at 4 °C. Wells were blocked with 100 µl filter sterilised BSA at 2 mg/ml in PBS for 1 h at 37 °C. Chondrocytes were isolated from normal and OA areas of adult knee joints as described above and cultured for 5-10 days. Cells were removed from flasks as above, centrifuged and extraction medium removed. After 2 washes in PBS, cells were re-suspended in 10 ml serum-free HAMs F12 medium. The cell suspension was filtered through a 70 micron cell sieve. The cell number and viability was determined. The cell suspension was made up to give  $1.0 \times 10^5$  cells per ml in serum-free HAMs F12 medium. 100 µl of cells were transferred to respective wells in test plate and incubated at 37 °C, for 3 hours. Wells were washed gently twice with PBS using a multichannel pipette, taking care not to touch or scrape the bottom of the wells with the tips as this could remove adherent cells. 100 µl of 4 % formalin in PBS was added to each well and cells allowed to fix for 1 h. The plate was centrifuged at 1000 rpm for 5 min to ensure that all adherent cells remain in wells and the formalin discarded. 100 µl of pre-filtered 1 % methylene-blue in distilled water was added to each well for 30 min. The plate was washed several times in distilled water to remove excess blue dye. 100 µl of 0.1N HCl in distilled water was added to each well for 5 min and mixed thoroughly, ensuring all particulate matter was in even solution. The plate was read at 630 nm. The number of adherent cells is proportional to the amount of dye bound which is proportional to absorbance at 630 nm (**GRAPH 2.2.**). All experiments were carried out in triplicate.

**GRAPH 2.2.**



Chondrocytes were isolated from normal areas of articular cartilage obtained from the tibial plateaux and femoral condyles of 1 female (age 77 years) and 2 males (median age 72 years, range 58-86) and from OA areas of articular cartilage obtained from the tibial plateaux and femoral condyles of 2 females (median age 68, range 67-71 years) and 1 male (age 58 years). All experiments were carried out in triplicate. The number of adherent cells is proportional to the amount of dye bound which is proportional to absorbance at 630 nm. The values shown represent the mean of 3 experiments  $\pm$  SEM.

### **2.6.2. Monoclonal antibodies**

The cell suspension was pre-incubated with anti-HMPG antibody M28B5, anti- $\beta$ 1 integrin antibodies TS2/16 and P4C10 and irrelevant anti-CD3 antibody for 30 min at 4 °C before addition to the 96 well plate.

### **2.6.3. Inhibitors**

The cell suspension was pre-incubated with inhibitors for 30 min at 37 °C. The inhibitors were used at the following concentrations: genistein (gen) 40  $\mu$ M, staurosporine (st)  $2 \times 10^{-6}$  M, cytochalasin D (cd)  $1 \times 10^{-6}$  M, pertussis toxin (pt)  $1 \times 10^{-5}$  M. Cells were then incubated with antibody for 30 min at 37 °C before addition to the 96 well plate.

### **2.6.4. Statistical analysis**

The variance of the two groups was assessed by comparing the F ratio of the standard deviations for each pair of values. If the variance of the two groups was not significantly different, the Student pooled variance T test was used to compare the means of two groups of data, with small sample numbers. If there was a significant difference between the standard deviations of the two groups, the non parametric Mann Whitney U test was used to investigate the significance of results between groups, as no inferences are made about the population from which the sample was taken and analysis of numerically graded data is possible. Significant results correspond to p values less than 0.05.

## **2.7. TYROSINE PHOSPHORYLATION**

### **2.7.1. Stimulation of chondrocytes.**

Tissue culture 84 mm petri dishes (Nunc, Life Technologies UK) were coated with 10 µg/ml FN or CVI overnight at 37 °C. Non specific binding sites were blocked with 1 mg/ml BSA for 3 h at 37 °C. Dishes to capture antibody were pre-coated with rabbit anti-mouse IgG at a dilution of 1:500 in PBS overnight at 37 °C. Antibodies TS2/16, KZ1 or M28B5 were added at 10 µg/ml for 3 h at 37 °C and then the plates were blocked with BSA as above. 5-10 day old chondrocytes taken from normal adult articular cartilage were removed from flasks, centrifuged and washed twice in PBS. Cells were re-suspended in 10 ml serum-free F12 medium, counted and viability determined to be greater than 95% by trypan blue exclusion.  $1 \times 10^6$ /ml cells were plated onto the coated petri dishes and allowed to adhere at 37 °C for specified periods of time, before protein extraction for assessment of protein tyrosine phosphorylation.

### **2.7.2. Assessment of protein tyrosine phosphorylation**

Cells were lysed by addition of 0.5 ml 2x lysis buffer for 20 min at 4 °C. Insoluble material was removed by centrifugation, lysates were transferred to microcentrifuge tubes and microcentrifuged for 15 min at high speed at 4 °C. Supernatants were dissolved in electrophoresis sample buffer and SDS-PAGE performed using slab gels with a gradient of 3-20 % acrylamide. Proteins were transferred to nitro-cellulose and incubated sequentially with primary mouse monoclonal anti-phosphotyrosine antibody, followed by biotinylated rabbit anti-mouse immunoglobulins then AP-conjugated AB complex. The colour development substrates 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and nitroblue tetrazolium (NBT) were used as visualising reagents. Alternatively anti-phosphotyrosine antibody directly conjugated to HRP was incubated with the nitro-cellulose at 1:1000 in 20 ml TBST for 1 h. The membrane was washed once for 15 min with TBST and twice for 5 min with TBST with rotation and the proteins visualised by ECL detection.



### 3.0. RESULTS

Raw data can be found in appendix IV

### 3.1. EXPRESSION OF NG2/HMPG IN HUMAN CARTILAGE *IN VIVO*

#### 3.1.1. Immunohistochemistry

NG2/HMPG expression in human cartilage was assessed by immunohistochemistry. Samples of normal articular cartilage were obtained from the patellae, tibial plateaux and femoral condyles of 1 female (age 67 years) and 7 males (median age 71 years, range 53 - 88). Samples of osteoarthritic articular cartilage were obtained from the patellae, tibial plateaux and femoral condyles of 13 males (median age 71 years, range 53 - 88). Fetal knee joints were obtained from 10 second trimester miscarriages (median age 17 weeks, range 14-20 weeks). NG2/HMPG was identified in both fetal and adult chondrocytes (TABLE 3.1.).

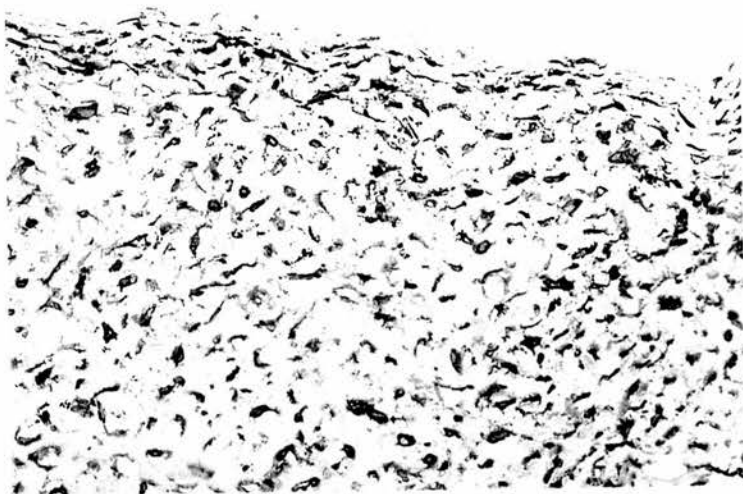
**TABLE 3.1.**

Immunoreactivity of human cartilage with anti-NG2/HMPG antibodies.  
All cases of fetal cartilage and cartilage from normal and OA adult joints showed strong immunostaining.

CARTILAGE	NO. SAMPLES	ANTI-NG2/HMPG (STANDARD DILUTION)
<b>Fetal</b>		
articular	10	++
epiphyseal	10	++
growth plate	10	++
<b>Adult</b>		
normal	8	++
OA	13	++

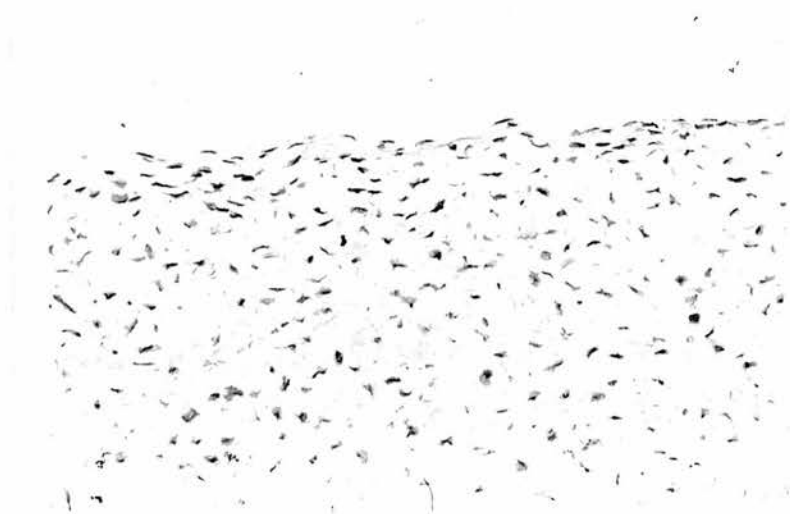
++ strong immunoreactivity

All cells in all cases of fetal cartilage showed similar staining patterns (**PLATES 3.1a-f.**). In each of 10 cases, chondrocytes in articular, epiphyseal and growth plate cartilage were strongly positive with antibodies against NG2 (N143.8) and HMPG (9.2.27, M28B5). Similarly, in cartilage from normal (8 cases) (**PLATE 3.2a.**) and osteoarthritic (13 cases) (**PLATE 3.2b.**) joints, strong immunostaining of chondrocytes was seen. There was no difference in immunoreactivity of sections removed from adult donors of different age or sex, from different areas of the knee joint (femoral condyle, tibial plateau or patella) or in chondrocytes at different positions within the cartilage (superficial or deep layer). In normal and OA cartilage the staining appears to be localised to the cell surface. In OA cartilage single chondrocytes and cell clusters both stain for NG2/HMPG.



**PLATE 3.1a.**

Articular cartilage from second trimester fetal knee joint stained with anti-HMPG antibody 9.2.27, showing strong immunoreactivity in all cells. Identical results were obtained with anti-HMPG antibody M28B5 and anti-NG2 antibody N143.8. Original magnification x50.



**PLATE 3.1b.**

Articular cartilage from second trimester fetal knee joint negative control, showing no immunoreactivity in the absence of antibody. Original magnification x50.



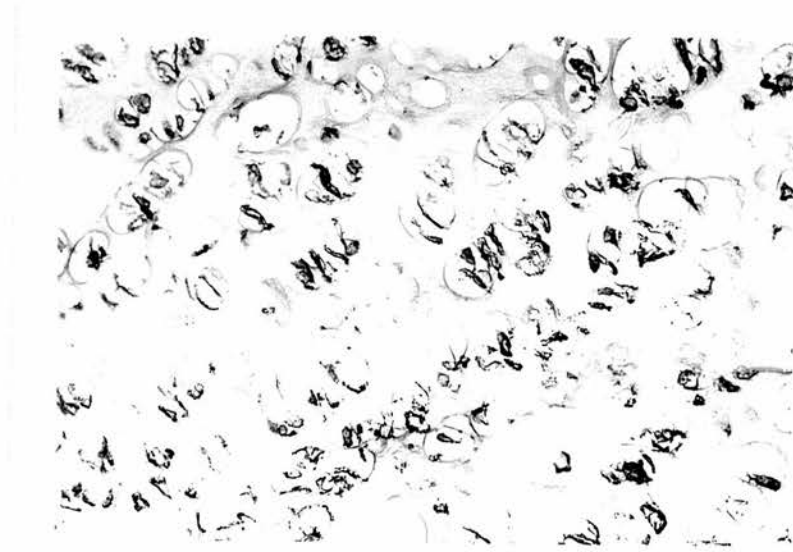
**PLATE 3.1c.**

Epiphyseal cartilage from second trimester fetal knee joint stained with anti-HMPG antibody 9.2.27, showing strong immunoreactivity in all cells. Identical results were obtained with anti-HMPG antibody M28B5 and anti-NG2 antibody N143.8. Original magnification x50.



**PLATE 3.1d.**

Epiphyseal cartilage from second trimester fetal knee joint negative control, showing no immunoreactivity in the absence of antibody. Original magnification x50.



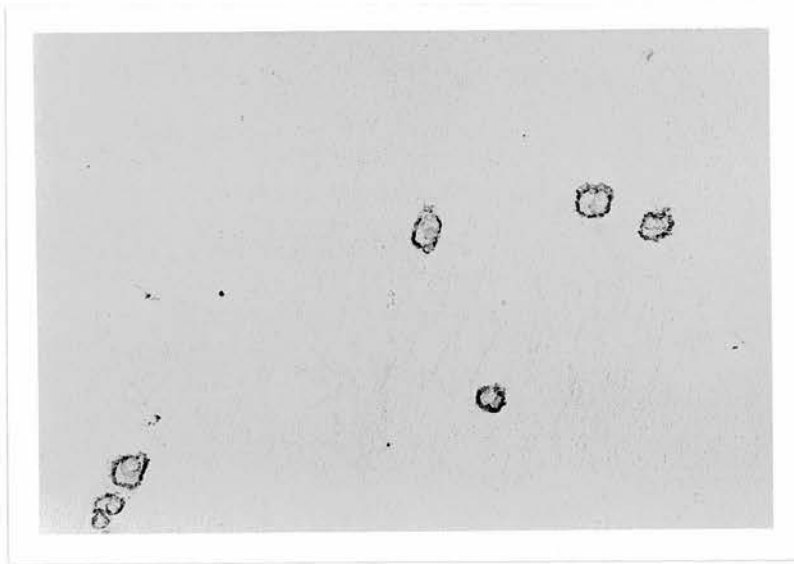
**PLATE 3.1e.**

Growth plate cartilage from second trimester fetal knee joint stained with anti-HMPG antibody 9.2.27, showing strong immunoreactivity in all cells. Identical results were obtained with anti-HMPG antibody M28B5 and anti-NG2 antibody N143.8. Original magnification x50.



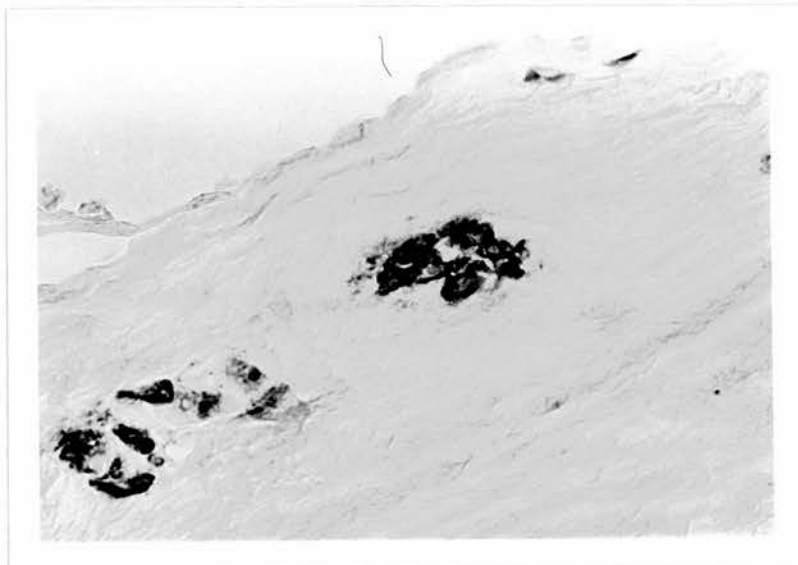
**PLATE 3.1f.**

Growth plate cartilage from second trimester fetal knee joint negative control, showing no immunoreactivity in the absence of antibody. Original magnification x50.



**PLATE 3.2a.**

Section of normal articular cartilage from knee joint of 67 year old female, immunostained with anti-HMPG antibody M28B5, showing NG2/HMPG localisation at the cell surface. Identical results were obtained with anti-HMPG antibody 9.2.27 and anti-NG2 antibody N143.8. Original magnification x50.



**PLATE 3.2b.**

Section of OA cartilage from knee joint of 71 year old male, immunostained with anti-HMPG antibody M28B5, showing NG2/HMPG positive cell clusters. Identical results were obtained with anti-HMPG antibody 9.2.27 and anti-NG2 antibody N143.8. Original magnification x50.

### **3.1.2. Quantification of immunohistology**

In normal cartilage the vast majority of chondrocytes were positive, but in OA cartilage a number of chondrocytes were found to be negative or showed only weak staining at standard dilutions of the antibodies used. To identify whether there was any difference in immunoreactivity of chondrocytes in normal and OA cartilage with anti-NG2/HMPG antibodies, sections were immunostained over a range of antibody dilutions. Samples of normal articular cartilage were obtained from the patellae, tibial plateaux and femoral condyles of 1 female (age 67 years) and 10 males (median age 71 years, range 53 - 88). Samples of mild osteoarthritic cartilage were obtained from the patellae, tibial plateaux and femoral condyles of 4 males (median age 61 years, range 53 - 69), samples of moderate osteoarthritic cartilage were obtained from the patellae, tibial plateaux and femoral condyles of 6 males (median age 64 years, range 53 - 74), and samples of severe osteoarthritic cartilage were obtained from the patellae, tibial plateaux and femoral condyles of 3 males (age 88 years). The results show that immunoreactivity is maintained in normal cartilage at lower dilutions than in OA cartilage (**TABLE 3.2.**). This suggests that expression of NG2/HMPG is lower in OA chondrocytes than normal chondrocytes or that antibody binding is restricted in sections of OA cartilage. There appeared to be no difference in immunoreactivity in sections removed from adult donors of different age and sex, from different areas of the knee joint (femoral condyle, tibial plateau or patella), in chondrocytes at different positions within the cartilage (superficial or deep layer) or single cells compared to clusters of cells in OA samples.



**TABLE 3.2.**

Immunoreactivity of chondrocytes in normal and osteoarthritic human cartilage over a range of anti-HMPG antibody dilutions.

Immunoreactivity is maintained in normal cartilage at lower dilutions than in OA cartilage.

CARTILAGE (NO. OF SAMPLES)	ANTI-HMPG (DILUTION)											
	<u>1:5</u>			<u>1:20</u>			<u>1:40</u>			<u>1:70</u>		
	++	+	-	++	+	-	++	+	-	++	+	-
<u>Normal</u> (11)	11	0	0	0	11	0	0	10	1	0	3	8
<u>OA</u>												
Mild (4)	4	0	0	0	4	0	0	3	1	0	0	4
Moderate (6)	3	3	0	0	5	1	0	0	6	0	0	6
Severe (3)	2	1	0	0	3	0	0	1	2	0	0	3

++ majority of cells stain positive

+ mixture of positive and negative cells

- no positive cells.

## **SUMMARY**

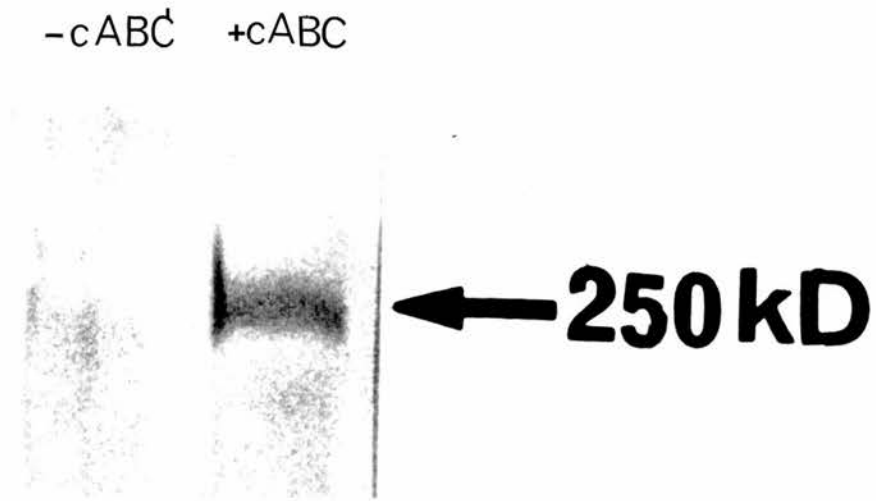
This is the first description of NG2/HMPG in human cartilage. It is not lost during maturation as in rat chondrocytes, but continues to be expressed in fully differentiated cells. It is also expressed by chondrocytes isolated from both normal and OA cartilage. Expression may be down regulated in chondrocytes from OA cartilage compared to chondrocytes from normal cartilage, or the NG2/HMPG epitope may become less accessible. This may be either as a result of a change in conformation or masking by interaction with another protein in OA cartilage.

## **3.2. EXPRESSION OF NG2/HMPG IN HUMAN CHONDROCYTES - *IN VITRO***

### **3.2.1. Western blotting**

It has been shown previously that NG2/HMPG is expressed by rat cell lines and human melanoma cells as both a CS rich PG of molecular weight greater than 400 kD, and a CS unmodified core protein which migrates at 250 kD (Bumol 1982, Stallcup 1990). Western blotting was carried out to establish whether the molecule expressed by human articular chondrocytes identified by histology has similar biochemical properties. Chondrocytes were isolated from normal cartilage obtained from the tibial plateaux, femoral condyles and patellae of 3 females (median age 71 years, range 66 - 75) and 9 males (median age 62 years, range 46 - 78).

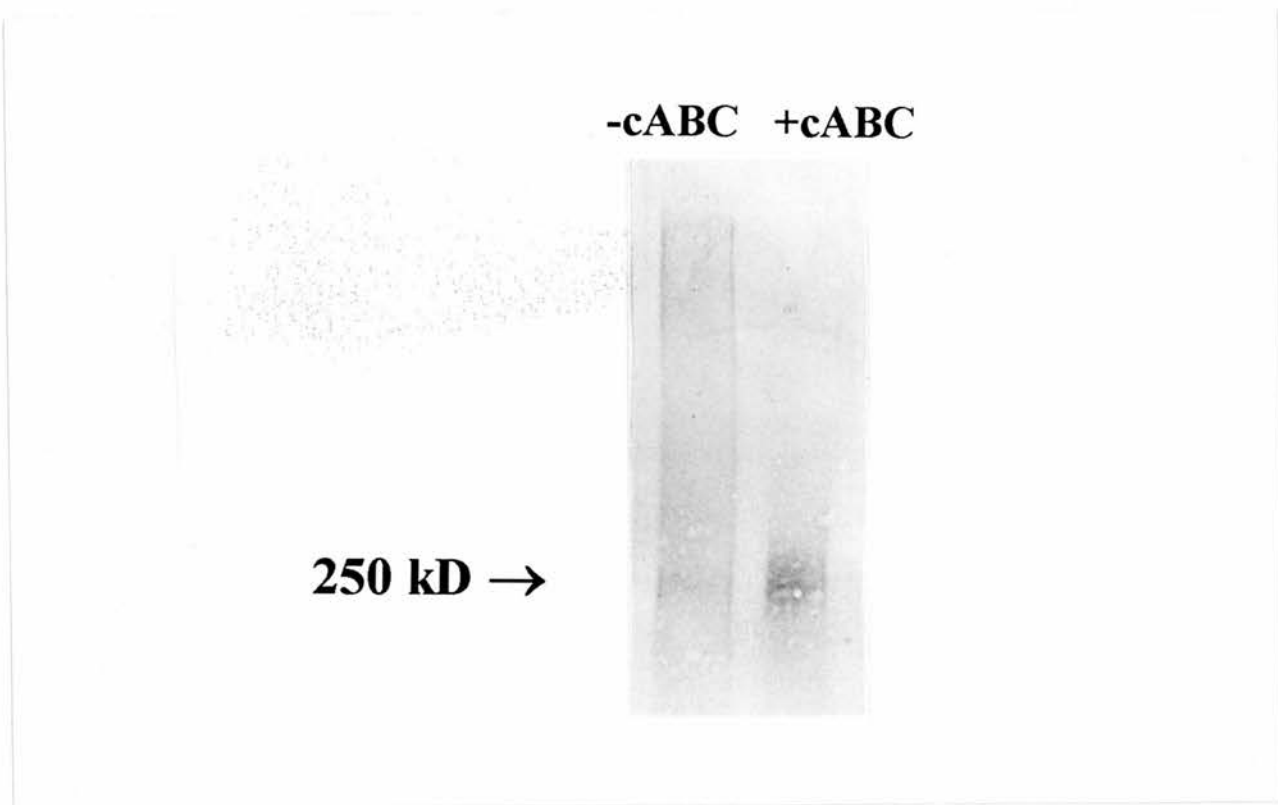
Chondrocytes were isolated from osteoarthritic cartilage obtained from the tibial plateaux, femoral condyles and patellae of 1 female (age 66 years) and 4 males (median age 64 years, range 50 - 78). Cells were lysed and the constituent proteins analysed by SDS PAGE and western blotting using two anti-HMPG monoclonal antibodies. Results with both antibodies were identical. Immunoblotting of extracts from untreated chondrocytes showed a smeared component of molecular weight greater than 400 kD and a faint band at 250 kD (**PLATE 3.3.**). When chondrocytes were incubated with chondroitinase ABC before protein extraction the 250 kD protein became predominant (**PLATE 3.3.**). This was seen in chondrocytes from both normal and OA cartilage. There is no difference in results from experiments using chondrocytes removed from donors of different age and sex or from different areas of the knee joint (femoral condyle, tibial plateau or patella).



**PLATE 3.3a.**

Western blot of lysates extracted from chondrocytes isolated from normal articular cartilage from the femoral condyle of a 78 year old male, probed with anti-HMPG antibody M28B5. Identical results were seen with anti-HMPG antibody 9.2.27.

Without chondroitinase ABC digestion (-cABC) a smeared component of molecular weight greater than 400 kD and a faint band at 250 kD are visible. The 250 kD protein becomes predominant upon digestion with chondroitinase ABC (+cABC).

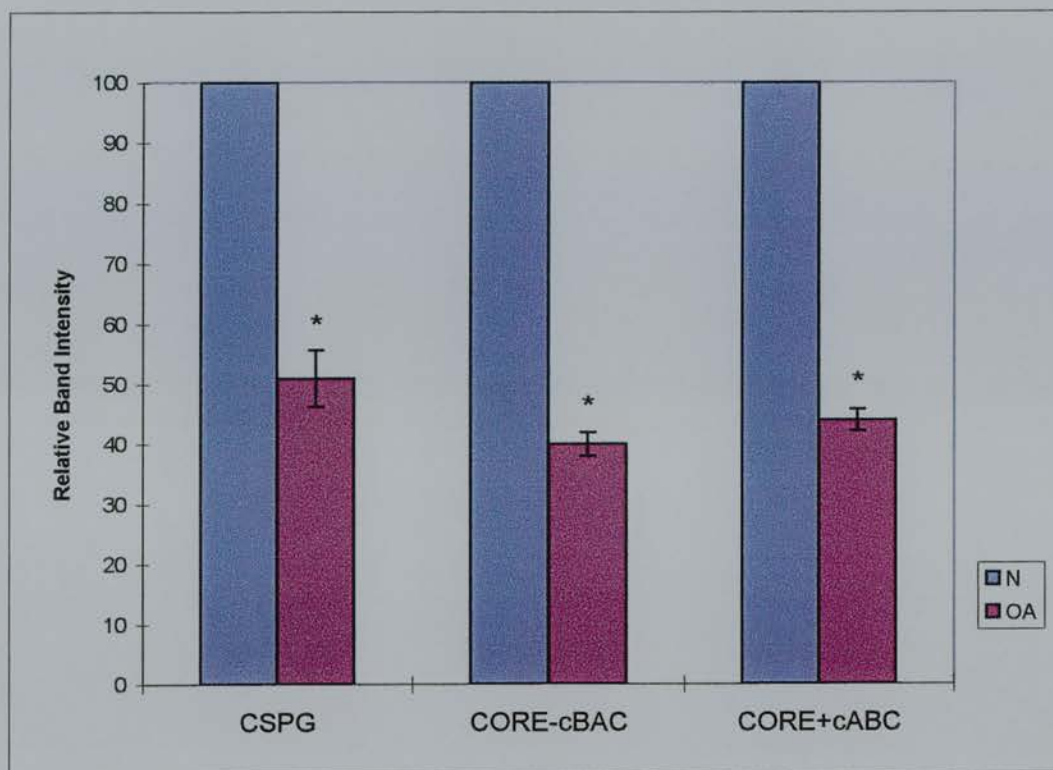


**PLATE 3.3b.**

Western blot of lysates extracted from chondrocytes isolated from OA articular cartilage from the femoral condyle of a 50 year old male, probed with anti-HMPG antibody M28B5. Identical results were seen with anti-HMPG antibody 9.2.27. Without chondroitinase ABC digestion (-cABC) a smeared component of molecular weight greater than 400 kD and a faint band at 250 kD are visible. The 250 kD protein becomes predominant upon digestion with chondroitinase ABC (+cABC).

### 3.2.2. Densitometry

To investigate whether chondrocytes isolated from normal cartilage express more NG2/HMPG than chondrocytes isolated from OA cartilage *in vitro*, equal amounts of total protein from extracts of cells were loaded onto gels. Chondrocytes were isolated from normal and OA articular cartilage obtained from the tibial plateaux and femoral condyles of 1 female (age 66 years) and 2 males (median age 70 years, range 61-78). After detection on western blots with anti-HMPG antibody M28B5, the intensity of each protein band was analysed. Quantitation of band intensity suggests that significantly less NG2/HMPG is present in chondrocytes isolated from OA cartilage compared to normal cartilage. In contrast FN expression is markedly upregulated in OA cartilage compared to normal cartilage (**GRAPH 3.1.**).

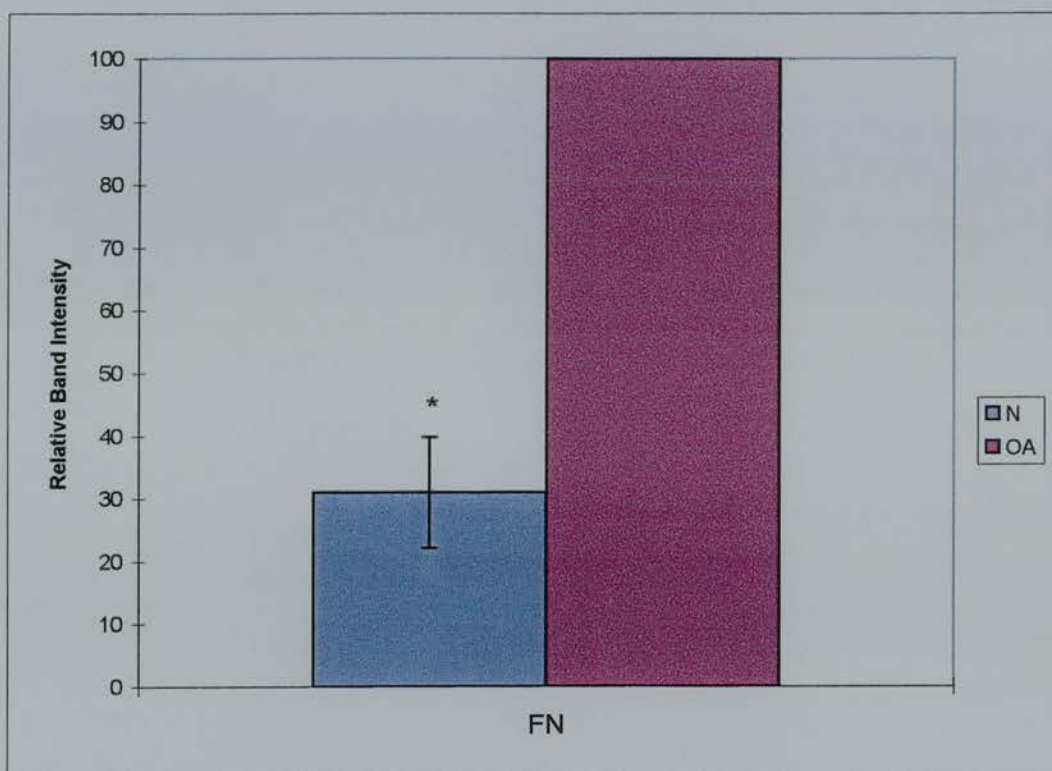


### GRAPH 3.1a.

Relative band intensities of NG2/HMPG from western blots of chondrocyte lysates. The band intensities of NG2/HMPG CSPG and core protein from untreated cells (-cABC) and core protein from cells treated with chondroitinase ABC (+cABC) are significantly greater in lysates of chondrocytes isolated from normal cartilage compared to OA cartilage. The values represent the mean of 3 experiments +/- the standard error of the mean (SEM).

\*  $p < 0.05$  (Mann Whitney U Test)





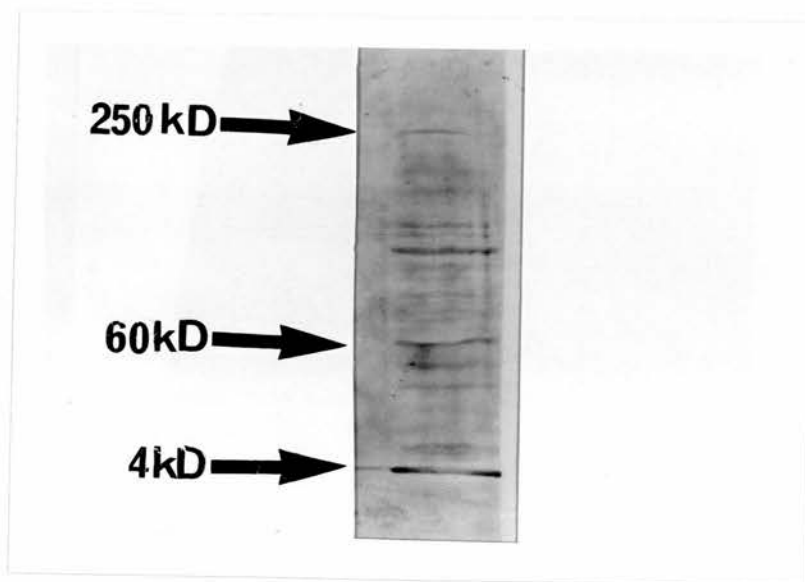
**GRAPH 3.1b.**

Relative band intensities of FN from western blots of chondrocyte lysates. The band intensity of FN is significantly greater in lysates of chondrocytes isolated from OA cartilage compared to normal cartilage. The values shown represent the mean of 3 experiments  $\pm$  SEM.

\*  $p < 0.01$  (Mann Whitney U Test)

### **3.2.3. Threonine phosphorylation**

Analysis of the primary structure of NG2/HMPG reveals three threonine residues, which together with surrounding amino acids form, potential PKC phosphorylation sites (Nishiyama 1991b, Pluschke 1996). Chondrocytes were isolated from normal articular cartilage obtained from the tibial plateaux and femoral condyles of 2 females (median age 70 years, range 65 - 74) and 3 males (median age 64 years, range 50 - 77). Western blots of chondrocyte lysates analysed with anti-phosphothreonine antibodies show that a wide range of proteins are phosphorylated on threonine, including one which migrates at 250 kD (**PLATE 3.4.**). The profile of proteins that are phosphorylated on threonine varies between samples, however the 250 kD protein is consistently phosphorylated in all samples. There is no difference in results from experiments using chondrocytes removed from donors of different age and sex or from different areas of the knee joint (femoral condyle, tibial plateau or patella). This supports the theory that NG2/HMPG may contain potential phosphorylation sites. Immunoprecipitation of NG2/HMPG before detection with anti-phosphothreonine antibodies will determine whether it is phosphorylated. Further work is needed to determine if NG2/HMPG is a substrate for PKC.



**PLATE 3.4.**

Western blot of chondrocyte lysates isolated from normal cartilage from the femoral condyle of a 74 year old female, probed with anti-phosphothreonine antibodies. A wide range of proteins are phosphorylated on threonine residues including one with a molecular weight of 250 kD.

## **SUMMARY**

NG2/HMPG is expressed by human articular chondrocytes in culture. Both the CSPG and the unmodified core protein are simultaneously expressed in chondrocytes isolated from both normal and OA cartilage. Lower band intensities of both the CSPG and unmodified core protein are detected in western blots using chondrocytes extracted from OA cartilage. This evidence supports the immunohistochemical data which suggests that less NG2/HMPG is expressed by OA cartilage.

### **3.3. FUNCTION OF NG2/HMPG IN HUMAN CHONDROCYTES - INTERACTION WITH ECM**

The best characterised interaction of NG2/HMPG is that with CVI. Anti-NG2 antibodies co-precipitate CVI and NG2 from rat cell lines, and the two proteins co-localise on the cell surface (Stallcup 1990). Rat cell lines exist which do not express NG2/HMPG and these cells secrete CVI from the cell surface (Nishiyama 1993). Transfection with NG2/HMPG cDNA results in NG2/HMPG expression by these cells and retention of CVI at the cell surface (Nishiyama 1993). Direct binding assays show that NG2/HMPG core protein interacts with CVI, indicating that NG2/HMPG acts as a cell surface receptor for CVI via a direct interaction between the two proteins (Burg 1996). Evidence also suggests that NG2/HMPG plays a role in human melanoma cell interaction with FN (Iida 1995). To attempt to demonstrate whether NG2/HMPG in human articular chondrocytes interacts with proteins of the ECM, the following experimental procedures were used.

#### **3.3.1. Immunoprecipitation**

Immunoprecipitation was used to detect whether NG2/HMPG is closely associated with any molecules at the surface of chondrocytes. Chondrocytes were isolated from normal articular cartilage obtained from the tibial plateaux and femoral condyles of 2 females (median age 74 years, range 56 - 89) and 2 males (age 74 years).

Immunoprecipitation of NG2/HMPG from normal adult chondrocytes with M28B5 resulted in a single band of molecular weight 250 kD (**PLATE 3.5.**). There is no difference in results from experiments using chondrocytes removed from donors of different age and sex or from different areas of the knee joint (femoral condyle, tibial plateau or patella).

**250kD→**

**60kD→**

**30kD→**

**PLATE 3.5.**

Autoradiograph showing band obtained after immunoprecipitation with protein A sepharose and anti-HMPG antibody M28B5 from radiolabelled chondrocyte lysates isolated from normal cartilage from the femoral condyle of a 74 year old male. No proteins are co-precipitated with NG2/HMPG. Identical results were seen using antibody anti-HMPG 9.2.27.

### 3.3.2. Immunofluorescence

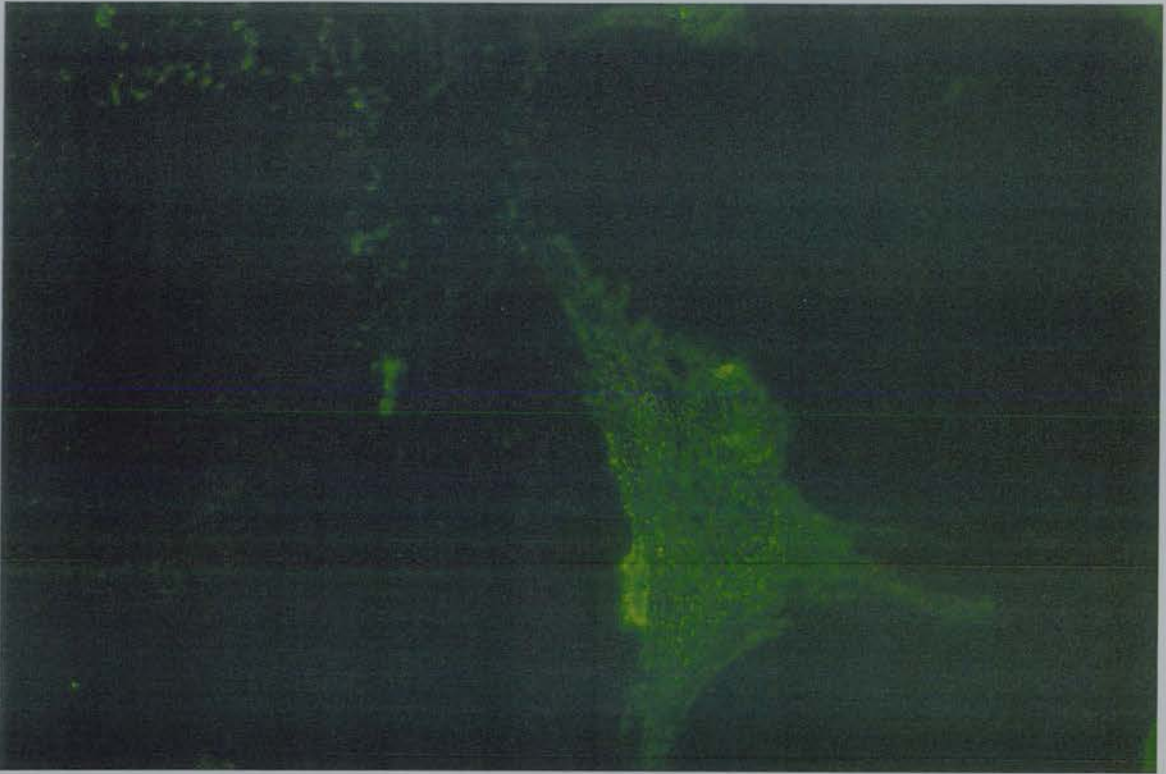
Immunofluorescence was used to study the distribution of NG2/HMPG at the chondrocyte cell surface and compare it to that of ECM proteins, in order to detect any co-localisation. Chondrocytes were isolated from normal articular cartilage obtained from the tibial plateaux and femoral condyles of 6 females (median age 72 years, range 59 - 84) and 8 males (median age 65 years, range 40 - 90).

Chondrocytes were isolated from OA articular cartilage obtained from the tibial plateaux and femoral condyles of 2 females (median age 60 years, range 59 - 61) and 3 males (median age 69 years, range 61 - 77). In chondrocytes isolated from normal adult cartilage *in vitro* NG2/HMPG was distributed in a punctate pattern (**PLATE 3.6a.**) similar to that described previously in human melanoma and rat cell lines (Bumol 1984, Stallcup 1984). Identical localisation was seen in chondrocytes isolated from OA adult cartilage (**PLATE 3.6b.**). The immunolocalisation of NG2/HMPG was compared to that of a number of putative extracellular ligands by double immunofluorescence staining. Chondrocytes were isolated from normal articular cartilage obtained from the tibial plateaux and femoral condyles of 2 females (median age 76 years, range 67 -84) and 1 male (age 82 years). The immunolocalisation of NG2/HMPG was different from that of a number of putative extracellular ligands, including FN, CII and CVI which are distributed in a fibrillar pattern at the cell surface (**PLATES 3.7a-f.**). There is no difference in results from experiments using chondrocytes removed from donors of different age and sex or from different areas of the knee joint (femoral condyle, tibial plateau or patella).

### SUMMARY

No direct interaction between NG2/HMPG and CVI has been demonstrated in chondrocytes using immunoprecipitation and immunofluorescence, as has previously been detected in rat cell lines (Stallcup 1990), nor does NG2/HMPG appear to co-localise with or bind to any ligands tested.

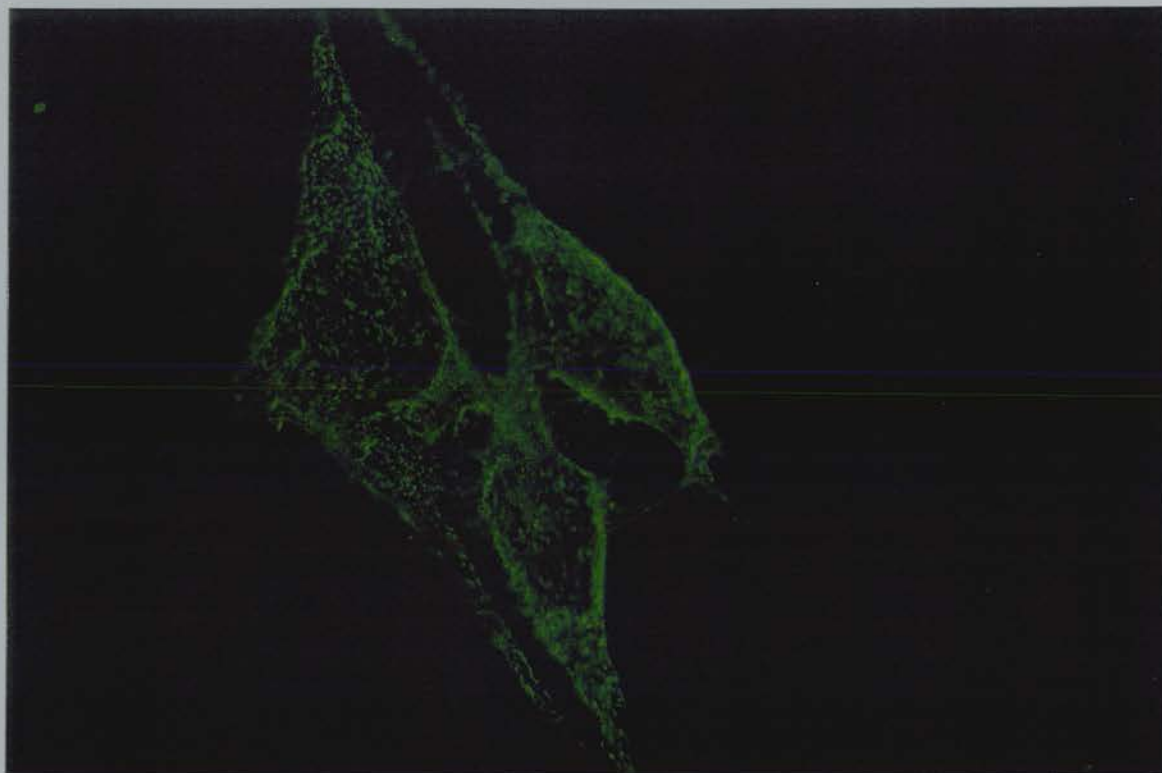




**PLATE 3.6a.**

Immunofluorescent localisation of NG2/HMPG.

Staining of chondrocytes isolated from normal cartilage obtained from the femoral condyle of a 90 year old male with anti-HMPG antibody M28B5 shows that NG2/HMPG is distributed in a punctate pattern. Identical results were obtained using anti-HMPG antibody 9.2.27.



**PLATE 3.6b.**

Immunofluorescent localisation of NG2/HMPG.

Staining of chondrocytes isolated from OA cartilage obtained from the femoral condyle of a 59 year old female with anti-HMPG antibody M28B5 shows that NG2/HMPG is distributed in a punctate pattern. Identical results were obtained using anti-HMPG antibody 9.2.27.



**PLATE 3.6c.**

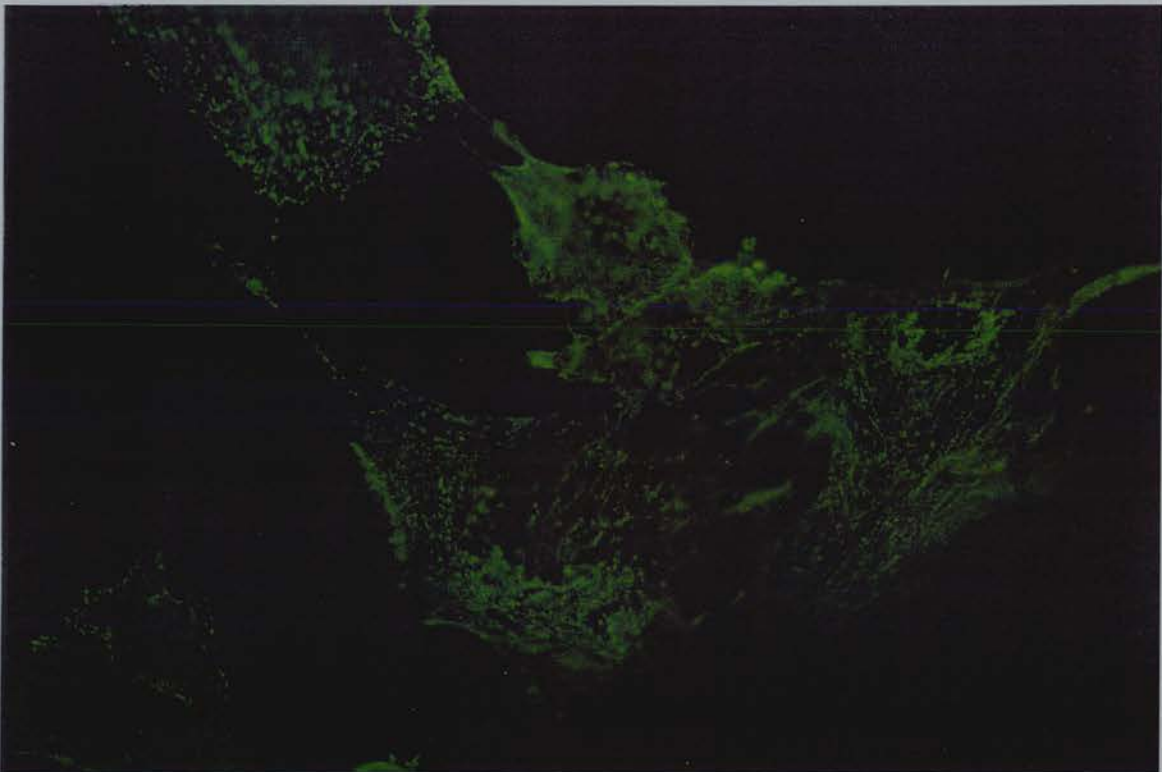
Negative control obtained by omission of primary antibody. No immunoreactivity is observed in the absence of antibody in chondrocytes isolated from normal cartilage isolated from the femoral condyle of a 90 year old male.

### **PLATES 3.7a-f.**

Double immunofluorescent localisation of NG2/HMPG and ECM proteins associated with chondrocytes.

Chondrocytes were isolated from normal cartilage obtained from the femoral condyle of 82 year old male showing that NG2/HMPG is distributed in a punctate pattern, whereas FN, CII and CVI are distributed in a filamentous pattern at the cell surface.

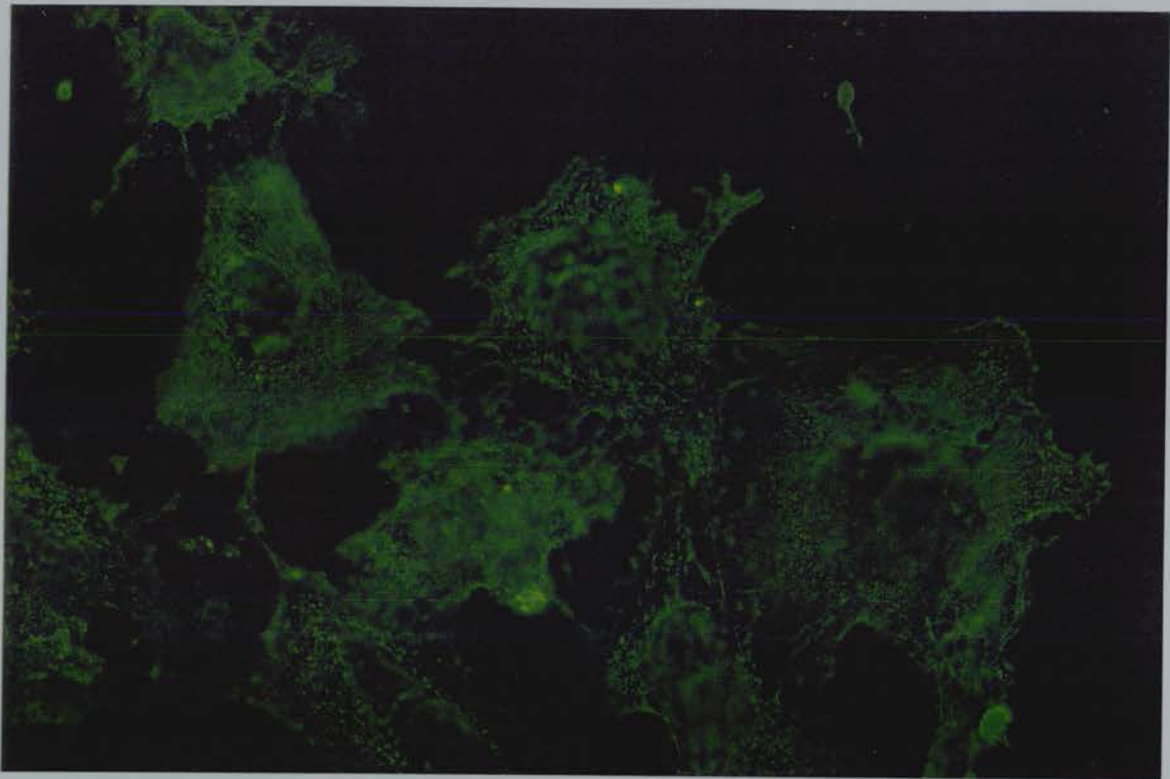
(a) NG2/HMPG



(b) FN



(c) NG2/HMPG

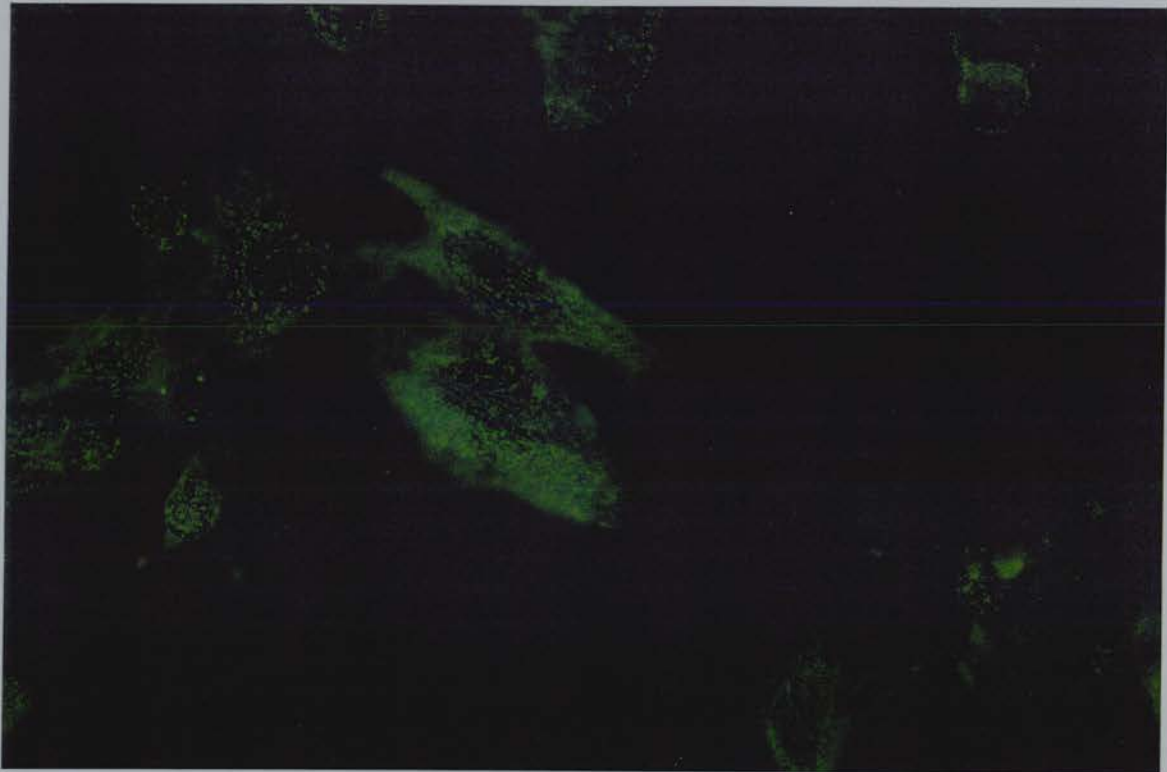


(d) CII

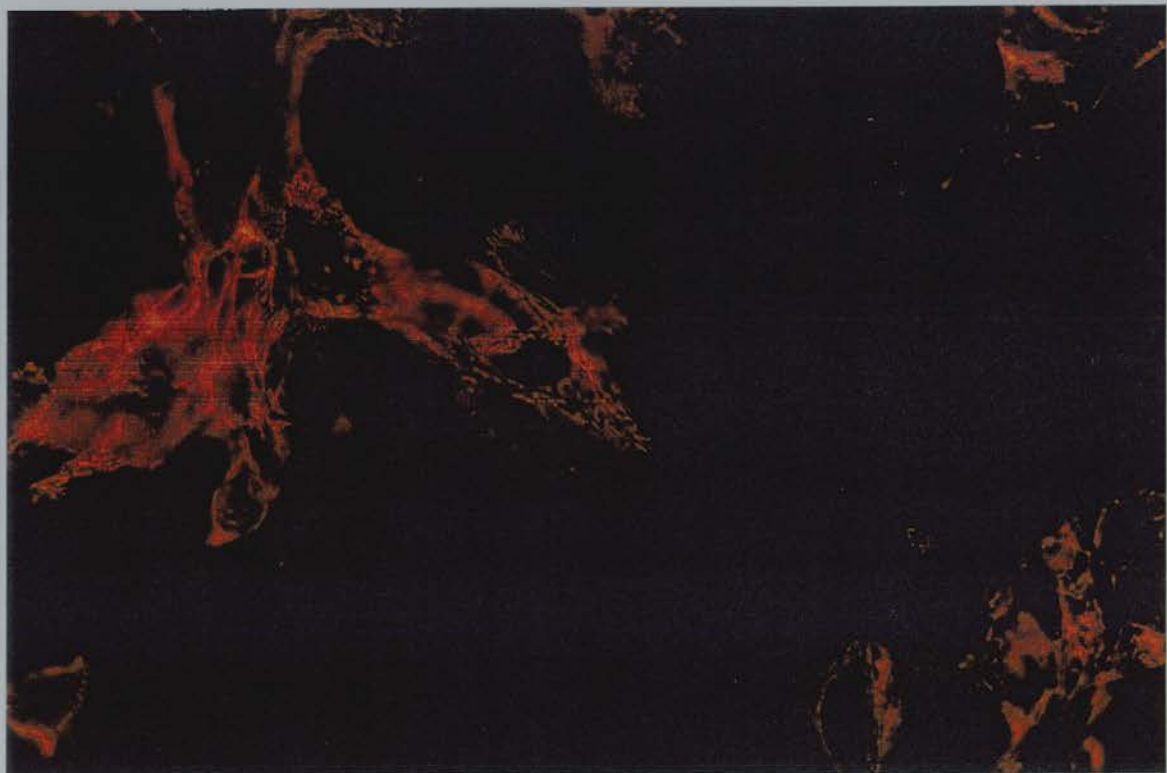




(e) NG2/HMPG



(f) CVI



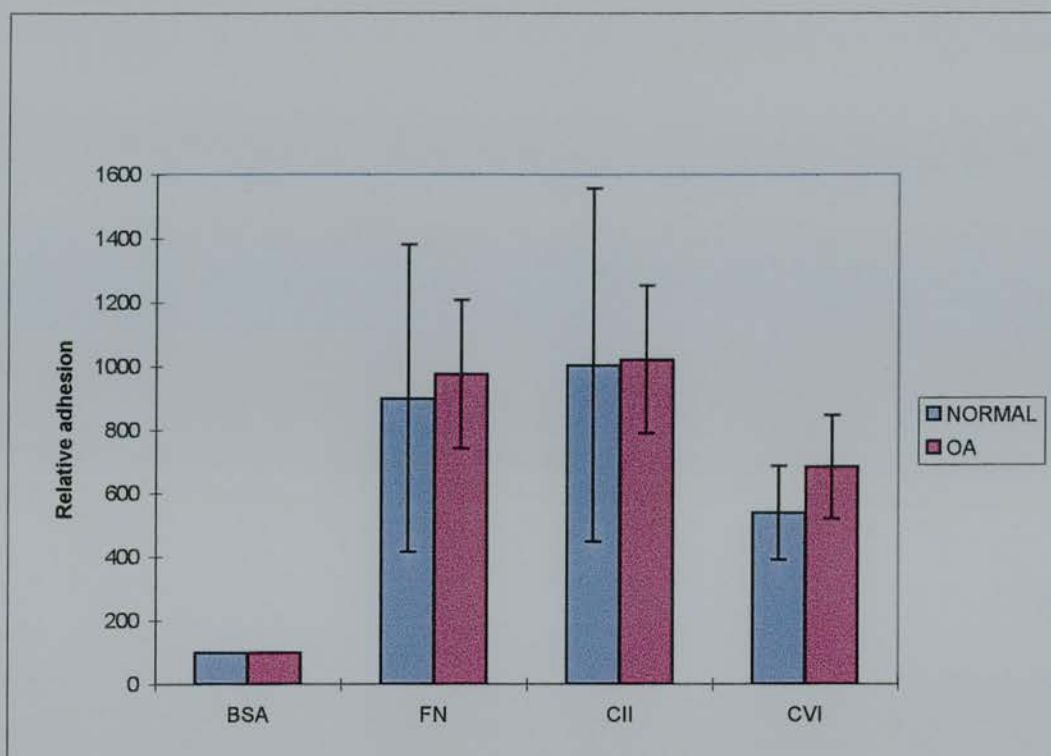


### 3.3.3. Cell Adhesion assays

Adhesion assays were used to characterise chondrocyte adhesion to ECM proteins FN, CII and CVI, and to study the effect of anti-HMPG antibodies on this interaction.

#### *Chondrocyte adhesion to ECM proteins.*

Chondrocytes extracted from normal and OA cartilage were allowed to adhere to ECM proteins FN, CII and CVI. Chondrocytes were isolated from normal cartilage obtained from the tibial plateaux and femoral condyles of 2 females (median age 71 years, range 65 - 77) and 2 males (median age 67 years, range 58 - 76) and from OA cartilage obtained from the tibial plateaux and femoral condyles of 3 females (median age 68 years, range 65 - 71) and 1 male (age 58 years). All experiments were carried out in triplicate. Non specific binding to BSA occurred at low levels. Both chondrocytes isolated from normal and OA cartilage adhere to FN, CII and CVI. There was no significant difference in the adhesion of chondrocytes to either FN, CII or CVI. There was no significant difference in the adhesion of chondrocytes isolated from normal and OA cartilage to FN, CII and CVI. (**GRAPH 3.2.**)



**GRAPH 3.2.**

Adhesion of chondrocytes extracted from normal and OA cartilage to FN, CII and CVI.

The values shown represent the mean of 4 experiments carried out in triplicate  $\pm$  SEM. There is no significant difference in the adhesion of chondrocytes isolated from normal cartilage to either FN, CII or CVI. Likewise there is no significant difference in the adhesion of chondrocytes isolated from OA cartilage to either FN, CII or CVI. (Mann Whitney U Test). There is no significant difference in the adhesion of chondrocytes isolated from normal cartilage compared to the adhesion of chondrocytes isolated from OA cartilage, to FN, CII or CVI (Mann Whitney U Test).

### *Effect of antibodies.*

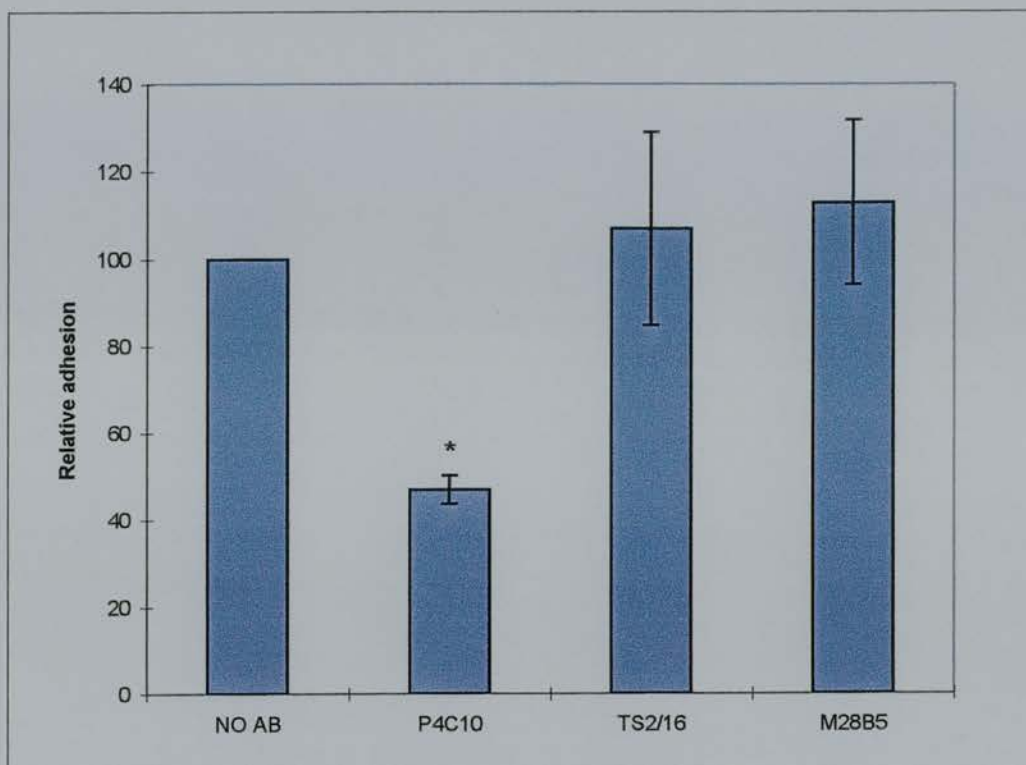
Anti-HMPG antibodies have been shown to modulate melanoma cell adhesion and spreading on FN (Iida 1995). The effect of anti-HMPG antibody M28B5 on chondrocyte adhesion to FN, CII and CVI was studied. Monoclonal antibodies against  $\beta 1$  integrin were used as positive controls. P4C10 inhibits attachment of cells to ECM proteins (Carter 1990) and TS2/16 stimulates cell attachment to ECM ligands (Arroyo 1992). Chondrocytes were isolated from normal articular cartilage obtained from the tibial plateaux and femoral condyles of 1 female (age 77 years) and 2 males (median age 71 years, range 65 - 77). Chondrocytes were isolated from osteoarthritic articular cartilage obtained from the tibial plateaux and femoral condyles of 1 female (age 77 years) and 2 males (median age 57 years, range 49 - 65).

In the absence of antibody chondrocytes isolated from normal cartilage adhere to FN. P4C10 significantly inhibits this adhesion by 53 %. Treatment of cells with TS2/16 results in a small but not statistically significant (7 %) stimulation of adhesion. Treatment of cells with M28B5 results in a small but not statistically significant (13 %) stimulation of adhesion. (**GRAPH 3.3.**)

In the absence of antibody chondrocytes isolated from normal cartilage adhere to CII. P4C10 significantly inhibits this adhesion by 59 %. Treatment of cells with TS2/16 results in a small but not statistically significant (7 %) stimulation of adhesion. Treatment of cells with M28B5 results in a small but not statistically significant (15 %) stimulation of adhesion. (**GRAPH 3.4.**)

In the absence of antibody chondrocytes isolated from normal cartilage adhere to CVI. P4C10 significantly inhibits this adhesion by 61 %. Treatment of cells with TS2/16 results in a significant (35 %) stimulation of adhesion. Treatment of cells with M28B5 results in a significant (26 %) stimulation of adhesion. (**GRAPH 3.5.**)

The adhesion of chondrocytes isolated from OA cartilage to ECM molecules FN, CII and CVI was studied in the same way. P4C10 significantly inhibited adhesion to FN by 37 %, to CII by 65 % and to CVI by 59 %. TS2/16 and M28B5 have a small but insignificant stimulatory effect on adhesion to FN, CII and CVI (**GRAPH 3.6., 3.7. and 3.8.**).



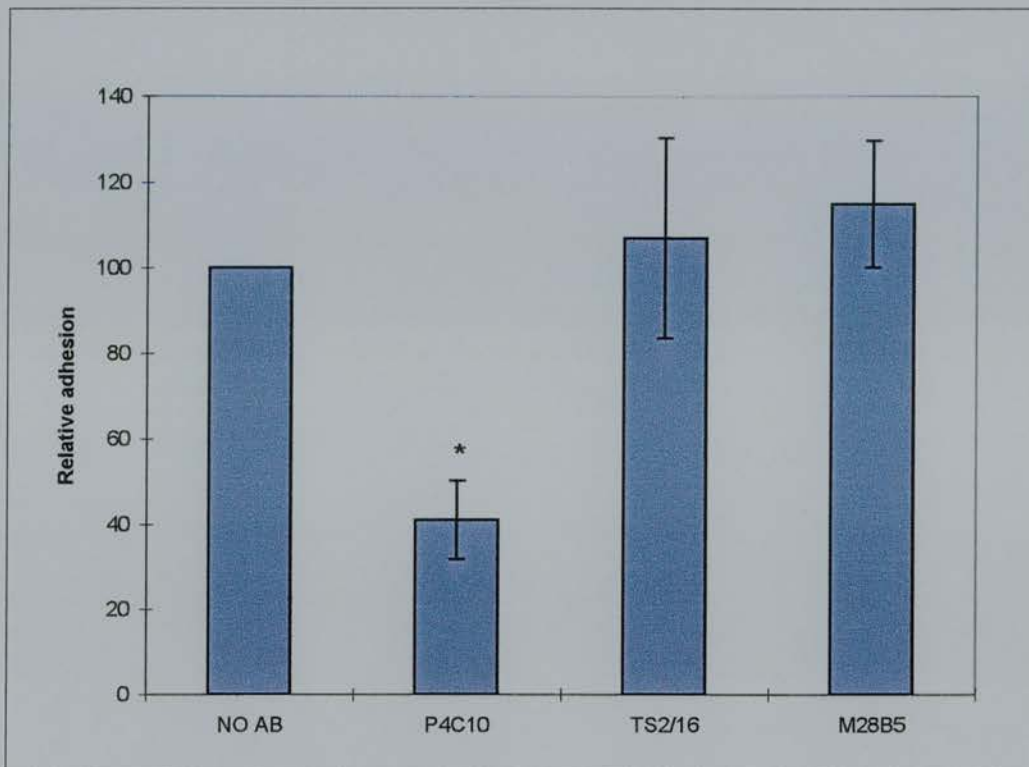
### GRAPH 3.3.

Adhesion of chondrocytes to FN - effect of anti  $\beta 1$  integrin and HMPG antibodies.

The adhesion of chondrocytes isolated from normal cartilage to FN in the absence of antibodies was compared to adhesion of cells treated with monoclonal antibodies for 30 min at 4°C. The values shown represent the mean of 3 experiments carried out in triplicate  $\pm$  SEM.

In the absence of antibody chondrocytes adhere to FN. P4C10 significantly inhibits this adhesion. Treatment of cells with TS2/16 and M28B5 results in a small but not statistically significant stimulation of adhesion to FN.

\*  $p < 0.05$  (Mann Whitney U Test)



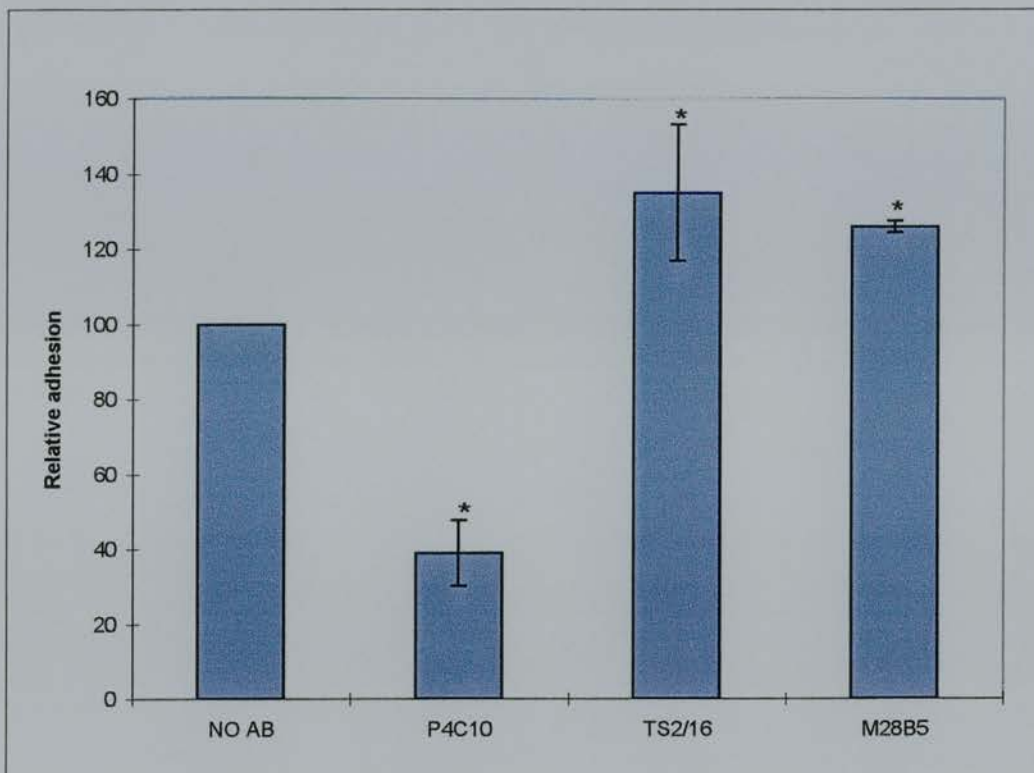
#### GRAPH 3.4.

Adhesion of chondrocytes to CII - effect of anti  $\beta 1$  integrin and HMPG antibodies.

The adhesion of chondrocytes isolated from normal cartilage to CII in the absence of antibodies was compared to adhesion of cells treated with monoclonal antibodies for 30 min at 4°C. The values shown represent the mean of 3 experiments carried out in triplicate  $\pm$  SEM.

In the absence of antibody chondrocytes adhere to CII. P4C10 significantly inhibits this adhesion. Treatment of cells with TS2/16 and M28B5 results in a small but not statistically significant stimulation of adhesion to CII.

\*  $p < 0.05$  (Mann Whitney U Test)



### GRAPH 3.5.

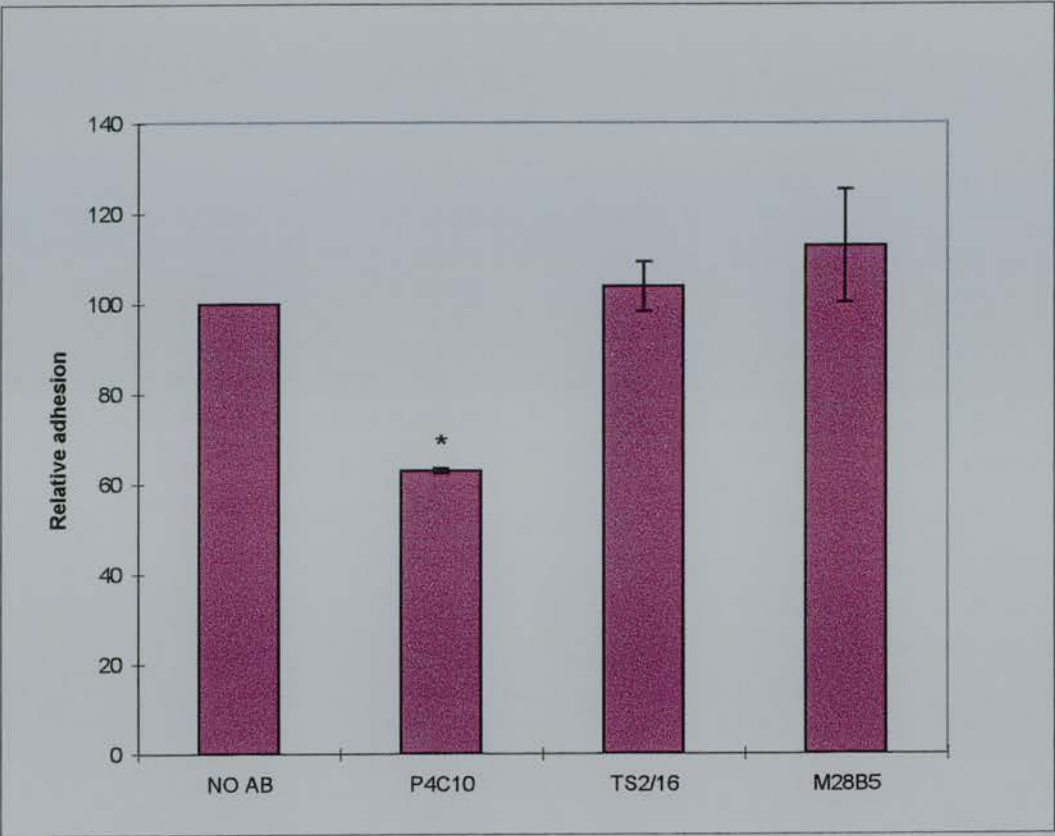
Adhesion of chondrocytes to CVI - effect of anti  $\beta 1$  integrin and HMPG antibodies.

The adhesion of chondrocytes isolated from normal cartilage to CVI in the absence of antibodies was compared to adhesion of cells treated with monoclonal antibodies for 30 min at 4°C. The values shown represent the mean of 3 experiments carried out in triplicate  $\pm$  SEM.

In the absence of antibody chondrocytes adhere to CVI. P4C10 significantly inhibits this adhesion. Treatment of cells with TS2/16 and M28B5 results in a significant stimulation of adhesion to CVI

\*  $p < 0.05$  (Mann Whitney U Test)





**GRAPH 3.6.**

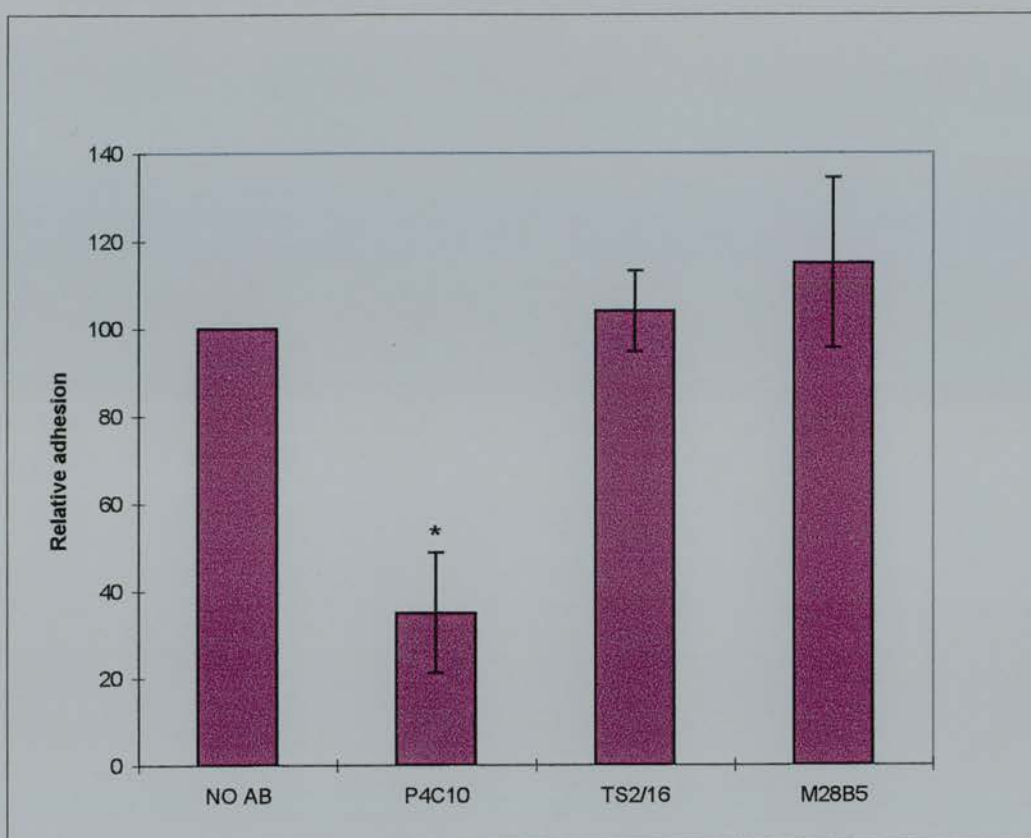
Adhesion of chondrocytes to FN - effect of anti  $\beta 1$  integrin and HMPG antibodies.

The adhesion of chondrocytes isolated from OA cartilage to FN in the absence of antibodies was compared to adhesion of cells treated with monoclonal antibodies for 30 min at 4°C. The values shown represent the mean of 3 experiments carried out in triplicate  $\pm$  SEM.

In the absence of antibody chondrocytes adhere to FN. P4C10 significantly inhibits this adhesion. Treatment of cells with TS2/16 and M28B5 results in a small but not statistically significant stimulation of adhesion to FN.

\*  $p < 0.05$  (Mann Whitney U Test)





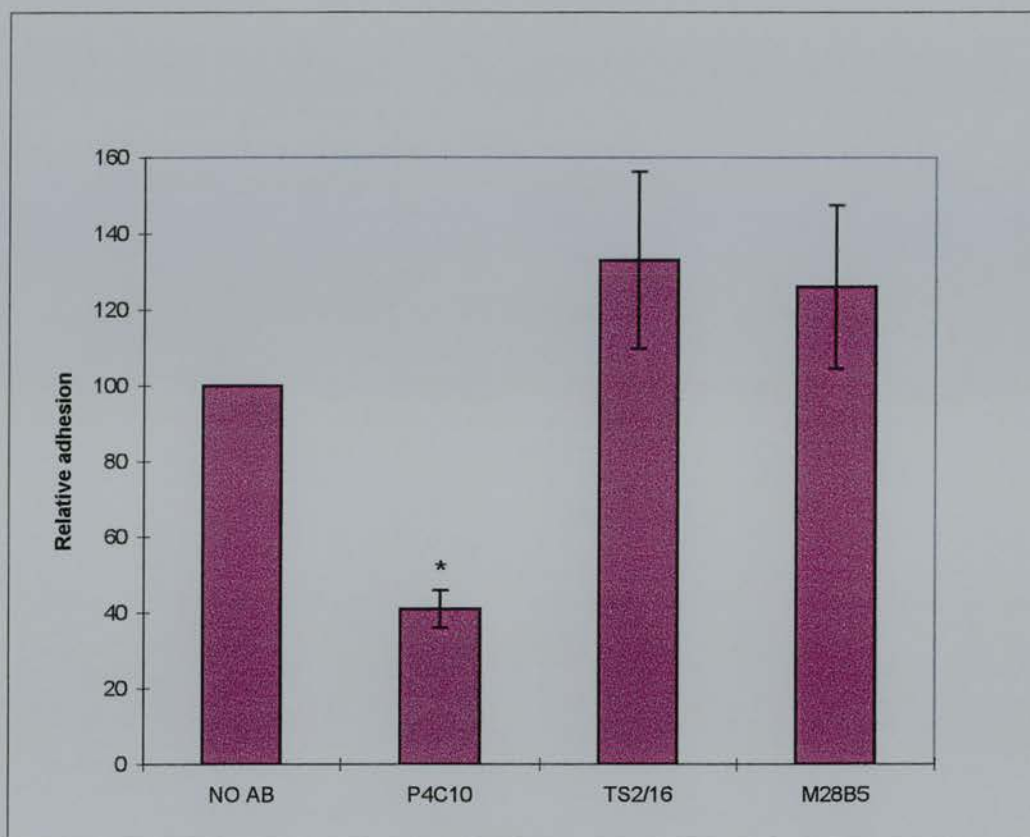
**GRAPH 3.7.**

Adhesion of chondrocytes to CII - effect of anti  $\beta 1$  integrin and HMPG antibodies.

The adhesion of chondrocytes isolated from OA cartilage to CII in the absence of antibodies was compared to adhesion of cells treated with monoclonal antibodies for 30 min at 4°C. The values shown represent the mean of 3 experiments carried out in triplicate  $\pm$  SEM.

In the absence of antibody chondrocytes adhere to CII. P4C10 significantly inhibits this adhesion. Treatment of cells with TS2/16 and M28B5 results in a small but not statistically significant stimulation of adhesion to CII.

\*  $p < 0.05$  (Mann Whitney U Test)



### GRAPH 3.8.

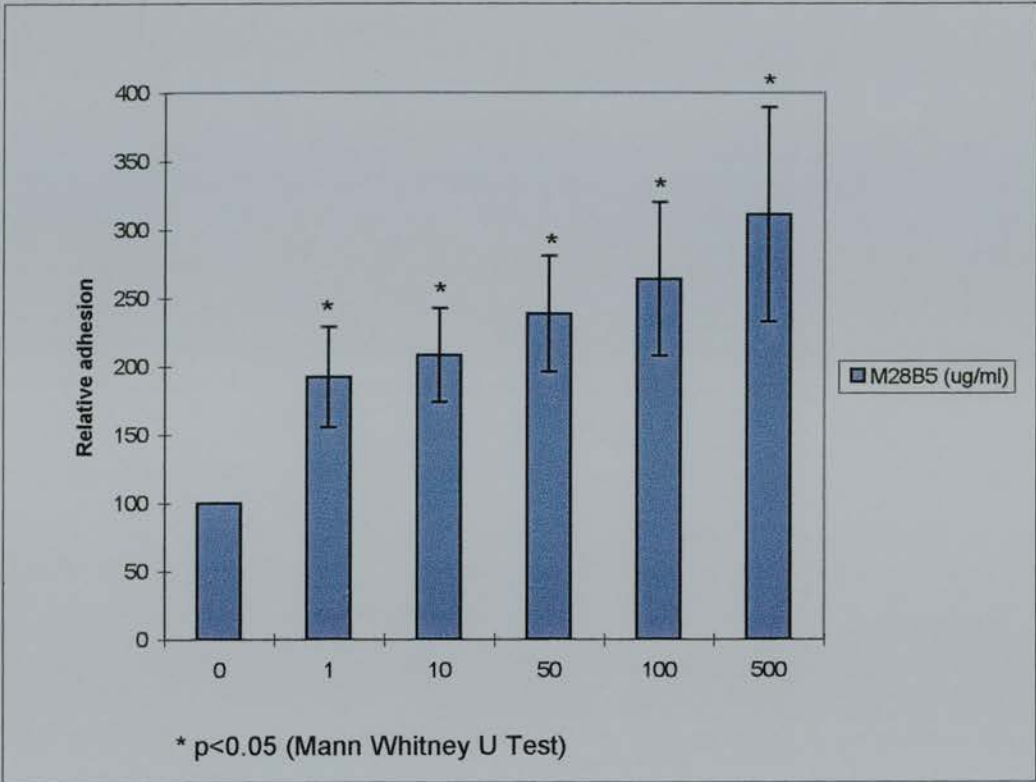
Adhesion of chondrocytes to CVI - effect of anti  $\beta 1$  integrin and HMPG antibodies. The adhesion of chondrocytes isolated from OA cartilage to CVI in the absence of antibodies was compared to adhesion of cells treated with monoclonal antibodies for 30 min at 4°C. The values shown represent the mean of 3 experiments carried out in triplicate  $\pm$  SEM.

In the absence of antibody chondrocytes adhere to CVI. P4C10 significantly inhibits this adhesion. Treatment of cells with TS2/16 and M28B5 results in a small but not statistically significant stimulation of adhesion to CVI.

\*  $p < 0.05$  (Mann Whitney U Test)

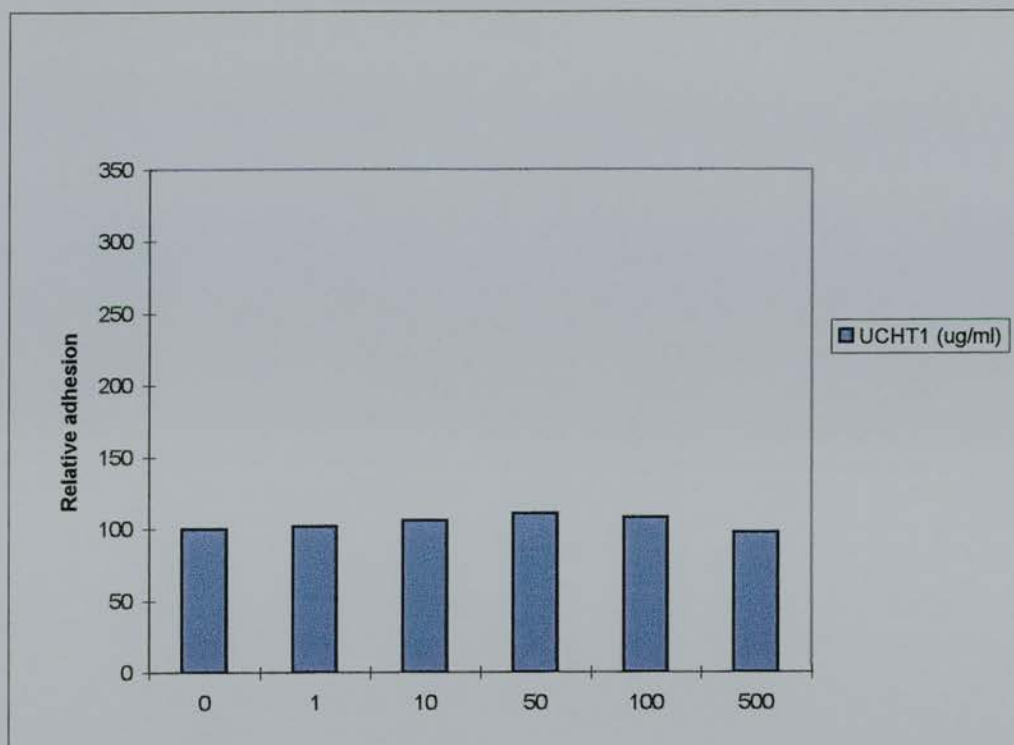
*Dose response effect of M28B5 antibody on chondrocyte adhesion to CVI.*

To demonstrate a dose response, chondrocytes were stimulated with increasing concentrations of M28B5. Chondrocytes were isolated from normal articular cartilage obtained from the tibial plateaux and femoral condyles of 1 female (age 74 years) and 2 males (median age 67 years, range 66 - 68). All experiments were carried out in triplicate. Stimulation of adhesion by M28B5 of chondrocytes isolated from normal cartilage to CVI increases in a dose dependent manner (**GRAPH 3.9.**). In contrast increasing concentrations of irrelevant antibody anti-CD3, UCHT-1, have no effect on adhesion to CVI (**GRAPH 3.10.**). Chondrocytes were obtained from normal articular cartilage obtained from the tibial plateaux and femoral condyles of 1 male (age 68 years). All experiments were carried out in triplicate.



**GRAPH 3.9.**

Effect of increasing concentrations of M28B5 on adhesion of chondrocytes to CVI. Stimulation of adhesion by M28B5 of chondrocytes isolated from normal cartilage to CVI increases in a dose dependent manner. The values shown represent the mean of 3 experiments carried out in triplicate +/- SEM.



**GRAPH 3.10.**

Effect of increasing concentrations of UCHT-1 on adhesion of chondrocytes to CVI.

The values shown represent the mean of 1 experiment carried out triplicate

Adhesion of chondrocytes isolated from normal cartilage is unaffected by increasing concentrations of UCHT-1.

## SUMMARY

Chondrocytes isolated from both normal and OA cartilage adhere to FN, CII and CVI. Function blocking anti- $\beta$ 1 integrin antibodies inhibit adhesion of chondrocytes isolated from both normal and OA cartilage to FN, CII and CVI. This confirms that  $\beta$ 1 integrins mediate chondrocyte interaction with these proteins (Enomoto 1993, Loeser 1997). The adhesion of chondrocytes isolated from normal cartilage to CVI is stimulated by anti-HMPG antibody M28B5 in a dose dependent manner. Anti-HMPG antibody M28B5 has no effect on chondrocyte binding to FN or CII. There is a small stimulation of adhesion that does not reach significance. Similarly anti-HMPG antibody M28B5 has no significant effect on the adhesion of chondrocytes isolated from OA cartilage to FN, CII or CVI. NG2/HMPG may be involved in modulating the interaction of chondrocytes isolated from normal cartilage with CVI, but this process is altered in chondrocytes isolated from OA cartilage.



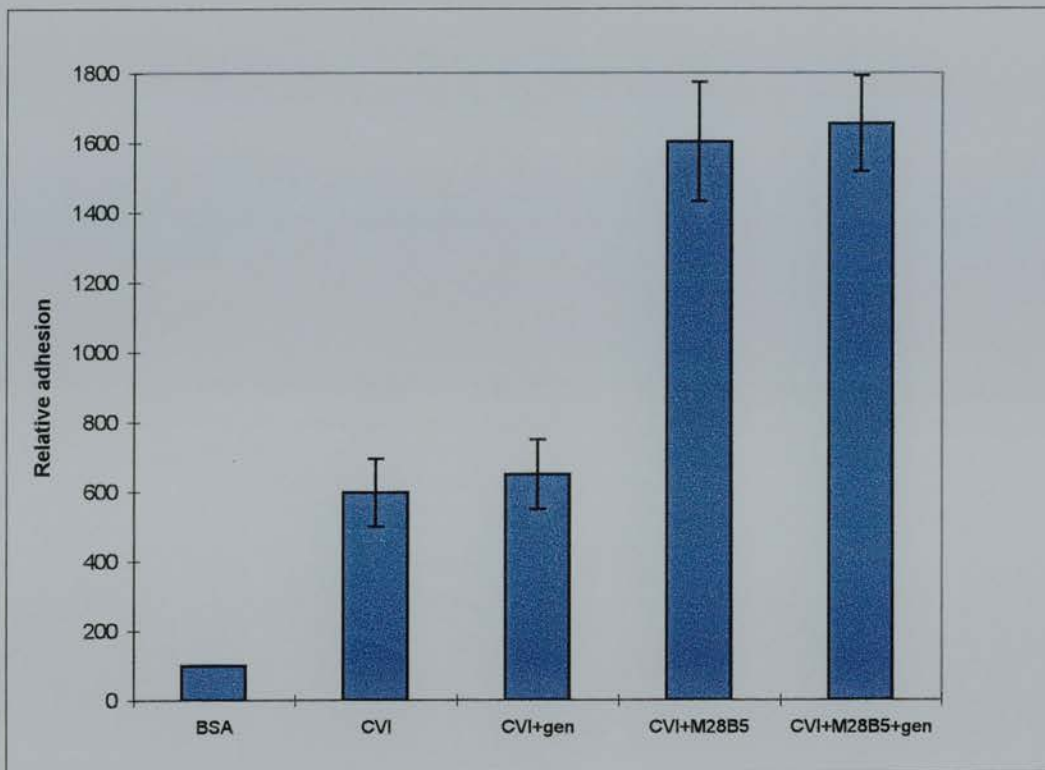
### **3.4. FUNCTION OF NG2/HMPG IN HUMAN CHONDROCYTES - SIGNAL TRANSDUCTION**

#### **3.4.1. Cell adhesion assays**

The work described previously in this study has provided no evidence for a direct interaction between NG2/HMPG and CVI as has been seen in rat cell lines (Stallcup 1990). However stimulation of NG2/HMPG in melanoma cells lines with anti-HMPG antibody indirectly affects cell interaction with FN. NG2/HMPG is thought to modulate  $\beta 1$  interaction with FN via inside out signalling (Iida 1995). Treatment of chondrocytes isolated from normal cartilage with M28B5 results in an increase in adhesion to CVI suggesting that NG2/HMPG has an albeit indirect but possibly important function in the regulation of chondrocyte adhesion to the ECM. Therefore the function of NG2/HMPG in the adhesion of chondrocytes isolated from normal cartilage to CVI was further investigated. To determine the intracellular events which occur during M28B5 stimulated chondrocyte adhesion to CVI, chondrocytes were allowed to adhere to CVI in the presence of antibody and inhibitors of various intracellular signalling molecules.

Chondrocytes were isolated from normal cartilage obtained from the tibial plateaux and femoral condyles of 1 female (age 89 years) and 2 males (median age 67 years, range 66 - 68). The adhesion to CVI of chondrocytes isolated from normal cartilage, treated with genistein, an inhibitor of tyrosine kinases, was compared to cells treated with both M28B5 and genistein. Genistein has no significant effect on chondrocyte adhesion to CVI in the absence of antibody or on the increased chondrocyte adhesion to CVI resulting from stimulation with M28B5 (**GRAPH 3.11.**). Staurosporine, which inhibits calcium dependent protein kinase C activity, also has no significant effect either on adhesion to CVI or the M28B5 stimulated adhesion to CVI (**GRAPH 3.12.**). Cytochalasin D, which disrupts the actin cytoskeleton, significantly inhibits both adhesion to CVI (61%) and M28B5 stimulated adhesion to CVI (82 %) (**GRAPH 3.13.**). Pertussis toxin, which inhibits  $G_i$  protein function, has no significant effect on adhesion to CVI in the absence of antibody but abolishes the stimulatory effect of M28B5 on adhesion to CVI (**GRAPH 3.14.**).



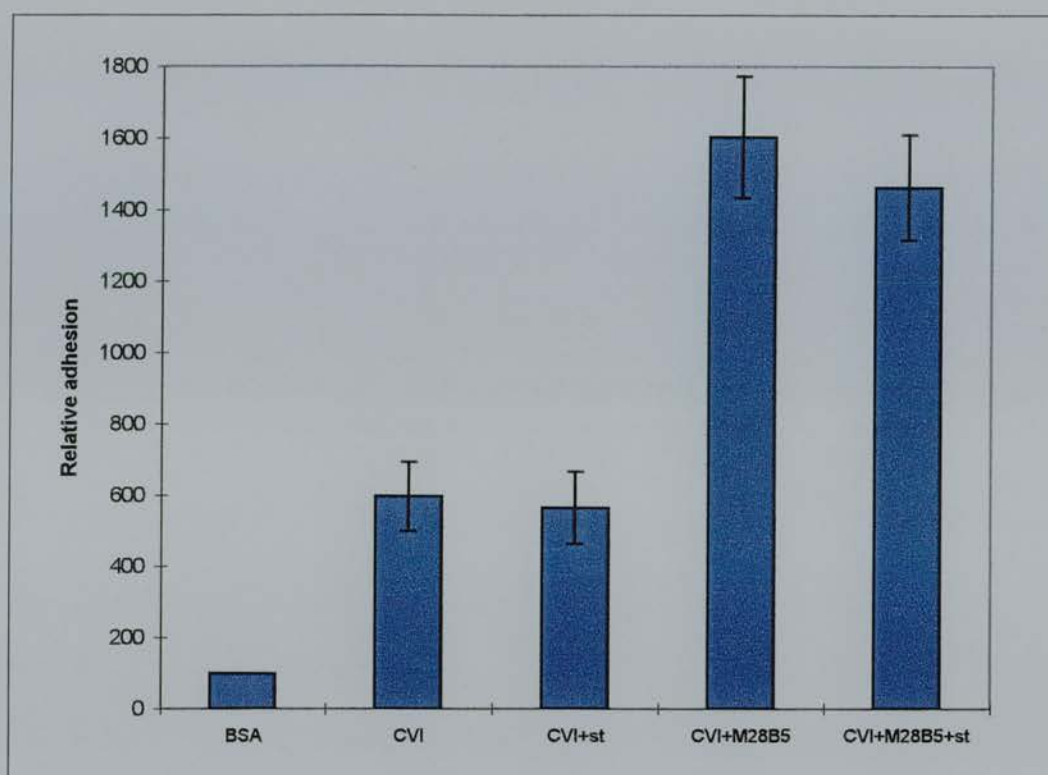


**GRAPH 3.11.**

Adhesion of chondrocytes to CVI - effect of genistein.

The adhesion of chondrocytes isolated from normal articular cartilage and treated with genistein for 30 min at 37 °C to CVI was compared to adhesion of chondrocytes not treated with inhibitor. In some cases cells were subsequently incubated with antibody M28B5 for 30 min at 37 °C. The values shown represent the mean of 3 experiments carried out in triplicate +/- SEM.

Non specific binding to BSA is minimal. Chondrocytes adhere to CVI. M28B5 stimulates chondrocyte adhesion to CVI. Genistein has no significant effect on chondrocyte adhesion to CVI or to the stimulated chondrocyte adhesion to CVI resulting from treatment with M28B5. (Student T Test)

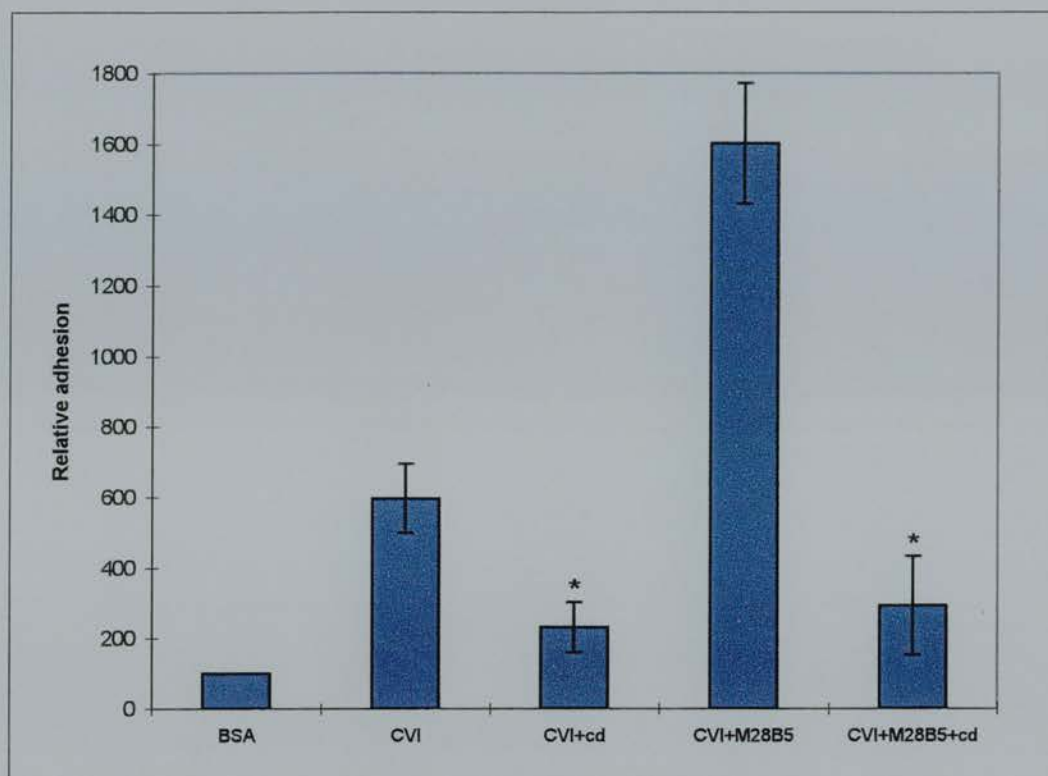


**GRAPH 3.12.**

Adhesion of chondrocytes to CVI - effect of staurosporine.

The adhesion of chondrocytes isolated from normal articular cartilage and treated with staurosporine for 30 min at 37 °C to CVI was compared to adhesion of chondrocytes not treated with inhibitor. In some cases cells were subsequently incubated with antibody M28B5 for 30 min at 37 °C. The values shown represent the mean of 3 experiments carried out in triplicate  $\pm$  SEM.

Non specific binding to BSA is minimal. Chondrocytes adhere to CVI. M28B5 stimulates chondrocyte adhesion to CVI. Staurosporine has no significant effect on chondrocyte adhesion to CVI or to the stimulated chondrocyte adhesion to CVI resulting from treatment with M28B5. (Student T Test)



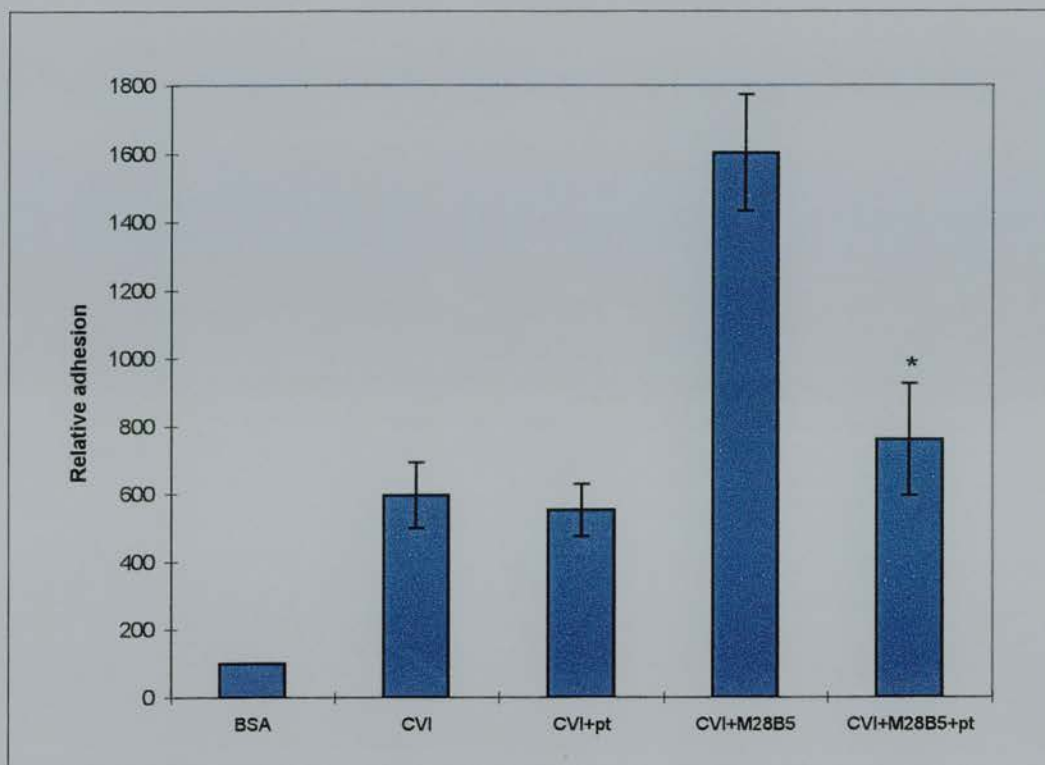
**GRAPH 3.13.**

Adhesion of chondrocytes to CVI - effect of cytochalasin D.

The adhesion of chondrocytes isolated from normal articular cartilage and treated with cytochalasin D for 30 min at 37 °C to CVI was compared to adhesion of chondrocytes not treated with inhibitor. In some cases cells were subsequently incubated with antibody M28B5 for 30 min at 37 °C. The values shown represent the mean of 3 experiments carried out in triplicate +/- SEM.

Non specific binding to BSA is minimal. Chondrocytes adhere to CVI. Cytochalasin D significantly inhibits adhesion to CVI. M28B5 stimulates chondrocyte adhesion to CVI. Cytochalasin D also significantly inhibits the stimulated chondrocyte adhesion to CVI resulting from treatment with M28B5.

\*  $p < 0.05$  (Student T Test)



**GRAPH 3.14.**

Adhesion of chondrocytes to CVI - effect of pertussis toxin.

The adhesion of chondrocytes isolated from normal articular cartilage and treated with pertussis toxin for 30 min at 37 °C to CVI was compared to adhesion of chondrocytes not treated with inhibitor. In some cases cells were subsequently incubated with antibody M28B5 for 30 min at 37 °C. The values shown represent the mean of 3 experiments carried out in triplicate +/-SEM.

Non specific binding to BSA is minimal. Chondrocytes adhere to CVI. Pertussis toxin has no significant effect on chondrocyte adhesion to CVI. M28B5 stimulates chondrocyte adhesion to CVI. Pertussis toxin abolishes stimulated adhesion to CVI caused by treatment with M28B5.

\*  $p < 0.05$  (Student T Test)

## SUMMARY

The adhesion of chondrocytes isolated from normal cartilage to CVI in the absence of M28B5 antibody is unaffected by inhibitors of tyrosine specific kinases, calcium dependent protein kinase C and Gi proteins, suggesting that these intracellular signalling molecules are not involved in chondrocyte adhesion to CVI.

Chondrocyte adhesion to CVI in the presence of antibody M28B5 is also unaffected by genistein and staurosporine but is inhibited by pertussis toxin. This suggests that the stimulated adhesion of chondrocytes to CVI does not involve tyrosine specific kinases and calcium dependent protein kinase C but does involve Gi protein mediated signalling.

Cytochalasin D inhibits chondrocyte adhesion to CVI in the absence or presence of M28B5, suggesting that the actin cytoskeleton plays a role in the adhesion of chondrocytes to CVI. However it is not possible to determine the extent of inhibition of stimulated adhesion by M28B5 specifically.

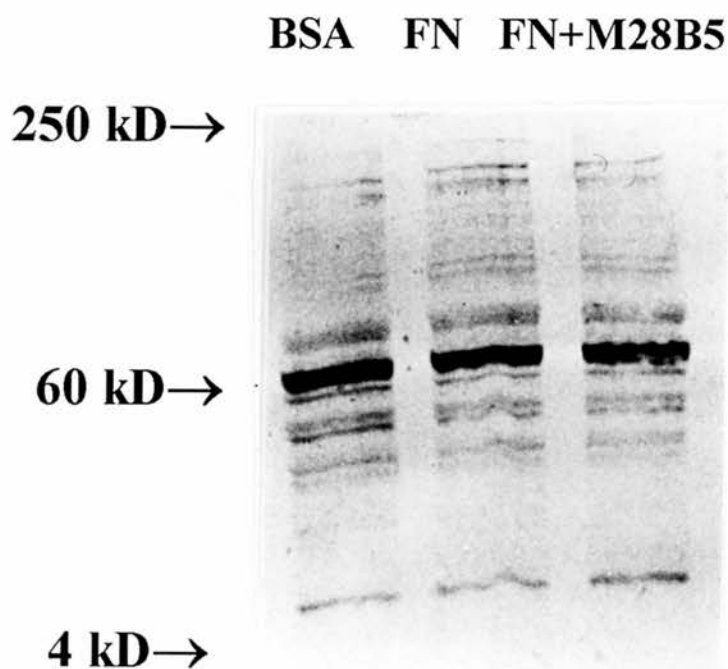


### 3.4.2. Tyrosine phosphorylation

Human melanoma cells allowed to adhere to a mixture of FN and 9.2.27, spread and form focal contacts, and two proteins become tyrosine phosphorylated (Iida 1995). To determine whether adhesion of chondrocytes to FN or a composite substrate of FN and M28B5 results in the stimulation of tyrosine kinase activity, chondrocytes were allowed to adhere to FN or M28B5 and FN for 1 h, after which time the profile of tyrosine phosphorylated proteins was compared to that of unstimulated cells allowed to adhere to BSA. Chondrocytes were isolated from normal articular cartilage obtained from the tibial plateaux and femoral condyles of 1 female (age 75 years) and 2 males (median age 55 years, range 52 - 60). Many proteins of a wide range of molecular weights are tyrosine phosphorylated, however no difference between untreated and stimulated cells was seen (**PLATE 3.8.**). The same was observed when cells were allowed to adhere to CVI, KZ1 or TS2/16 were compared to control cells. This suggests that adhesion of chondrocytes to these substrates for 1 h does not stimulate the activity of tyrosine specific kinases.

Cells were also allowed to adhere from 1 min to 1 h to anti-HMPG antibody M28B5 and no change in the profile of proteins that were phosphorylated on tyrosine residues was observed compared to chondrocytes that were plated on BSA, confirming that M28B5 alone does not stimulate tyrosine kinase activity over this time course.

There is no difference in results from experiments using chondrocytes removed from donors of different age and sex or from different areas of the knee joint (femoral condyle, tibial plateau or patella).



**PLATE 3.8.**

Western blot of chondrocyte lysates isolated from normal articular cartilage obtained from the femoral condyle of a 75 year old female, plated on dishes coated with BSA, FN or FN and M28B5 for 1 h. When probed with anti-phosphotyrosine antibodies identical phosphorylation profiles are seen. Identical results were seen when chondrocytes were stimulated with CVI, KZ1 and TS2/16 and when chondrocytes were allowed to adhere to M28B5 for 1 min, 5 min 15 min, 30 min or 1 h.



### **3.4.3. Interaction with actin**

In rat cell lines NG2/HMPG has been shown to co-localise with actin stress fibres (Lin 1996a). Disruption of the actin cytoskeleton with cytochalasin D results in a release of NG2/HMPG from the cell surface (Lin 1996a), suggesting that NG2/HMPG acts as a cytoskeletal anchor by binding to actin. Chondrocytes were isolated from normal articular cartilage obtained from the tibial plateaux and femoral condyles of 1 female (age 76 years) and 3 males (median age 59 years, range 40 - 77) and stained with both anti-HMPG antibody M28B5 and phalloidin. Double immunofluorescence labelling for NG2/HMPG and actin in human chondrocytes did not show any clear association between the two molecules (**PLATE 3.9.**). There is no difference in results from experiments using chondrocytes removed from donors of different age and sex or from different areas of the knee joint (femoral condyle, tibial plateau or patella).

### **3.4.4. Interaction with PDGF $\alpha$ receptor**

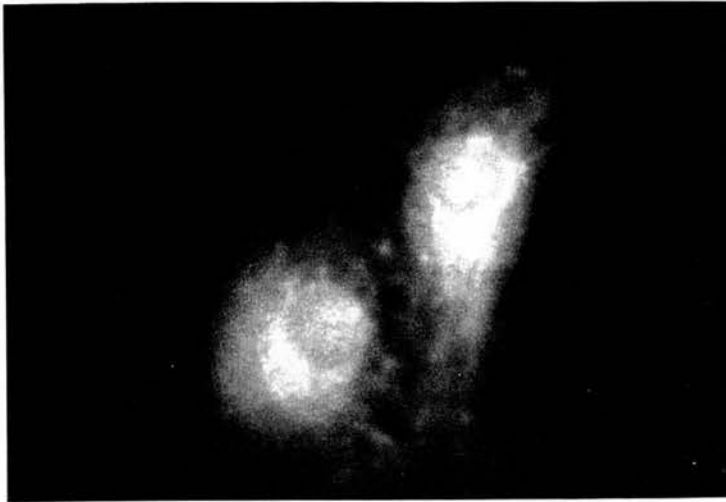
NG2/HMPG and PDGF $\alpha$ -R are expressed co-ordinately during the early stages of O-2A cell development and interaction between the two molecules is required for optimal response to PDGF (Nishiyama 1996a,b). Developing and adult rat vascular smooth muscle cells also require correct expression of NG2/HMPG and PDGF $\alpha$ -R to respond to PDGF (Grako 1995). Chondrocytes were isolated from normal articular cartilage obtained from the tibial plateaux and femoral condyles of 2 females (median age 65 years, range 61 - 69) and 1 male (age 61 years) and stained with antibodies against both HMPG and PDGF $\alpha$ -R. Adult articular chondrocytes express PDGF $\alpha$ -R (**PLATE 3.10a.**). Double immunofluorescence labelling for NG2/HMPG and PDGF  $\alpha$ -R in human chondrocytes did not show any clear association between the two molecules (**PLATE 3.10b,c.**). There is no difference in results from experiments using chondrocytes removed from donors of different age and sex or from different areas of the knee joint (femoral condyle, tibial plateau or patella).

**PLATE 3.9.**

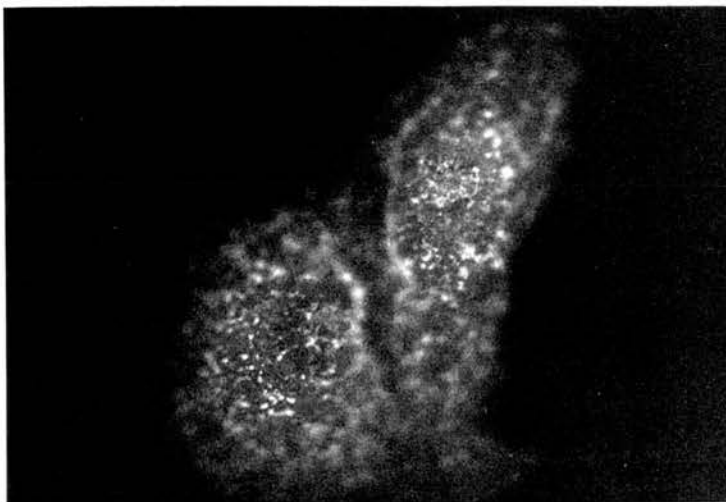
Immunofluorescent localisation of actin and NG2/HMPG.

Double labelling of chondrocytes isolated from normal articular cartilage obtained from the femoral condyle of a 76 year old female shows no clear association of the two molecules.

**(a)** actin



**(b)** NG2/HMPG

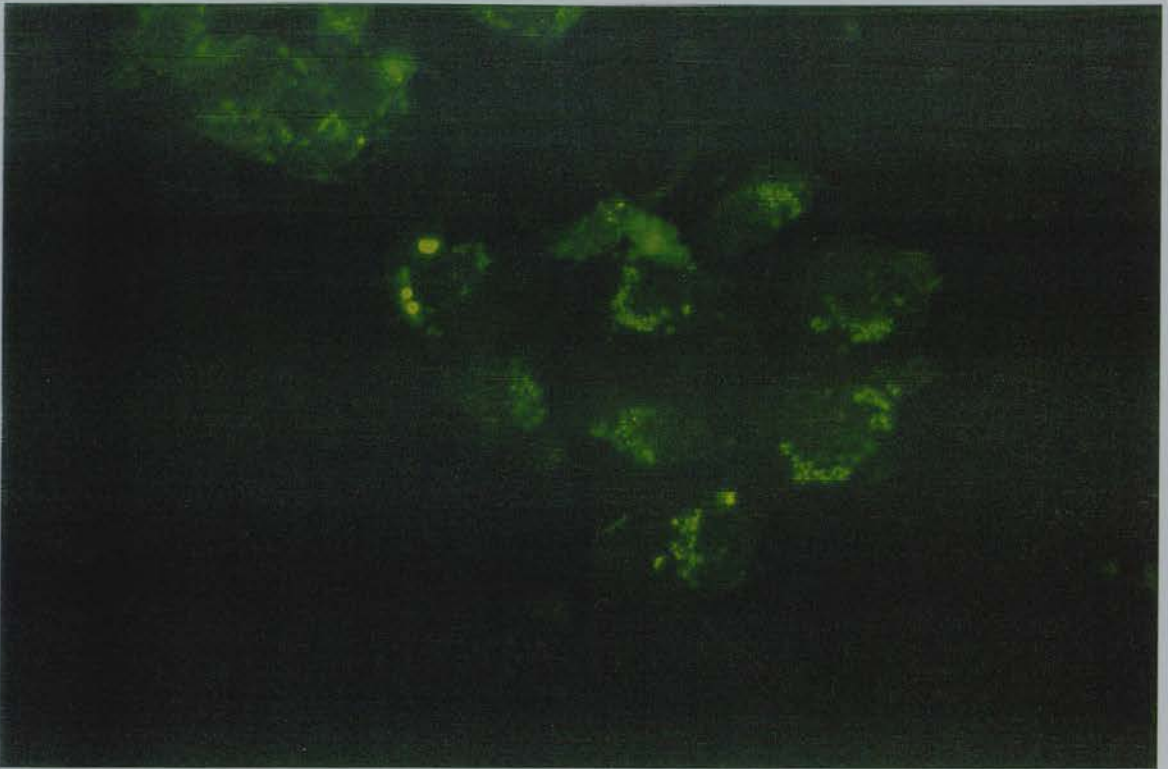


**PLATE 3.10.**

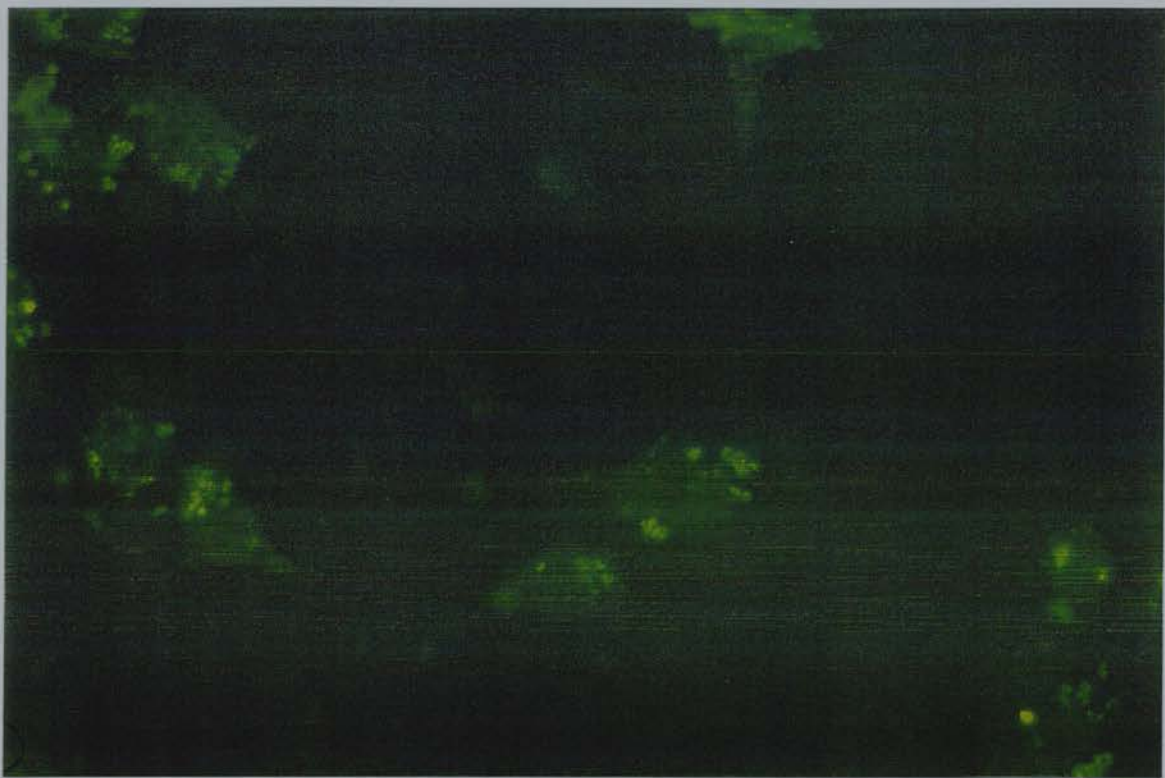
Immunofluorescent localisation of PDGF $\alpha$ -R and NG2/HMPG.

PDGF $\alpha$ -R is expressed by human articular chondrocytes isolated from normal cartilage obtained from the femoral condyle of a 61 year old male (a). Double labelling of chondrocytes isolated from normal cartilage obtained from the femoral condyle of a 61 year old male for PDGF $\alpha$ -R and NG2/HMPG shows no clear association of the two molecules (b, c).

(a) PDGF $\alpha$ -R



(b) PDGF $\alpha$ -R



(c) NG2/HMPG



## **4.0. DISCUSSION**

These studies have demonstrated that NG2/HMPG is expressed by human chondrocytes. It is present in both normal and OA adult articular cartilage, and in articular, epiphyseal and growth plate fetal cartilage. Expression is down regulated in chondrocytes isolated from OA cartilage. Biochemical analysis of NG2/HMPG in adult articular chondrocytes shows that it is expressed as the CS rich PG and the 250 kD protein core. These studies also provide evidence that NG2/HMPG is located in a distinct punctate pattern *in vitro* in chondrocytes isolated from normal and OA cartilage, and does not co-localise with any putative ligands such as actin, PDGF $\alpha$ -R, FN, CII and CVI. Anti-HMPG antibodies stimulate normal chondrocyte binding to CVI in a dose dependent manner, and this stimulation can be inhibited by pertussis toxin.

### **4.1. EXPRESSION OF NG2/HMPG BY HUMAN CHONDROCYTES**

Initial reports on NG2/HMPG show expression to be predominantly restricted to developing tissues. Expression was first described in rat cell lines with characteristics of both neural and glial cells (Stallcup 1981). NG2/HMPG is also expressed in O-2A glial progenitor cells of the rat cerebellum, although it ceases to be expressed following differentiation to mature oligodendrocytes (Stallcup 1987). In extra-neural tissues of rats, expression is also confined to developing tissues, in particular those of mesenchymal origin, including chondroblasts and smooth muscle progenitor cells (Nishiyama 1991a). NG2/HMPG expression has been studied in fetal and adult human tissue, and is found in malignant cells such as chondrosarcoma (Leger 1991) and melanoma (Reisfeld 1984, 1987), but not in several other carcinoma cell types, including lung and bladder, nor in normal cell types, including melanocytes, T and B cell lines or in normal tissues, including lung and colon (Reisfeld 1984, 1987). It therefore appears to have a limited distribution, suggesting a specialised function in cells.

Normal cartilage is rich in PGs, they constitute approximately 50% of the total tissue weight, of which the majority are chondroitin sulphated (Bayliss 1983).



In OA cartilage, chondrocytes assume a fetal phenotype, including increased proliferation and secretion of ECM molecules. This proceeds until the rate of reparative ECM protein synthesis is exceeded by the increase in ECM degradation and the tissue is destroyed (Poole 1993, Hamerman 1989).

The aim of this project was to determine whether NG2/HMPG is expressed in human cartilage by adult or fetal chondrocytes, and to compare expression in normal and OA cartilage. Immunohistochemical staining of human cartilage sections demonstrated that NG2/HMPG is expressed by fetal and adult normal cartilage *in vivo*. Expression is not down regulated once chondrocytes undergo terminal differentiation, as in the rat. NG2/HMPG is also expressed *in vivo* by chondrocytes from both normal and OA cartilage. However, immunohistological staining over a range of anti-HMPG antibody dilutions reveals that immunoreactivity is maintained at lower antibody concentrations in normal cartilage compared to OA cartilage. This suggests that expression of NG2/HMPG may be down regulated in OA tissue. Densitometric analysis of western blots of lysates of chondrocytes isolated from normal and OA cartilage probed with anti-HMPG antibodies show a down regulation of expression of NG2/HMPG in chondrocytes from OA cartilage. FN expression is known to be upregulated in OA cells (Burton-Wurster 1990), western blots of lysates of chondrocytes isolated from normal and OA cartilage probed with anti-FN antibodies show an increase in expression in chondrocytes isolated from OA cartilage. This *in vitro* work suggests that the decrease in immunoreactivity seen *in vivo* is due to a downregulation in expression of NG2/HMPG, and not due to inaccessibility of the NG2/HMPG epitope. The decrease in NG2/HMPG protein expression may result from degradation, secretion from the cell surface, or a change in the regulation or rate of synthesis. Breakdown products have been detected in rat cell lines, as has secreted NG2/HMPG (Nishiyama 1995), suggesting that it may be worthwhile to look for these forms of the protein in human chondrocytes. Alternatively, a down regulation in the synthesis of NG2/HMPG messenger RNA, or the rate of translation, may account for the lower levels of NG2/HMPG detected in OA cartilage, indicating that analysis of amounts of mRNA produced by normal and diseased tissue would be of interest.

NG2/HMPG expression in fetal and adult chondrocytes suggests that it may be important in chondrocyte function during development and throughout adulthood. The downregulation of NG2/HMPG expression observed in OA chondrocytes raises the possibility that it is important in the disease process. NG2/HMPG may play a regulatory role in the onset and progression of OA, the loss of NG2/HMPG may be a trigger for the initiation of osteoarthritis. Initial studies reveal that expression of NG2/HMPG is decreased even in mild cases of osteoarthritis, however it is unclear exactly when NG2/HMPG is lost from OA cartilage, or how this is regulated. Alternatively, the decrease in expression of NG2/HMPG may occur as a result of the imbalance in chondrocyte homeostasis that occurs in OA cartilage. An increased secretion of proteases and inability of chondrocytes to balance this destruction by increased PG synthesis, results in loss of PGs from the tissue. The significance of NG2/HMPG expression in normal and OA tissues remains unclear until more about its function is known.

## **4.2. BIOCHEMICAL PROPERTIES OF NG2/HMPG IN HUMAN CHONDROCYTES**

In cell types other than chondrocytes, NG2/HMPG is expressed as two forms simultaneously at the cell surface, both the CS rich PG and the unmodified protein core (Bumol 1982, Stallcup 1990). The role of the different forms, CS-rich and CS-free NG2/HMPG, has been addressed. In rat cell lines the protein core is responsible for mediating interactions with ECM ligands (Tillet 1997). However, CS GAG chains are essential for human melanoma cell interaction with FN (Iida 1992). Anionic GAG CS chains purified from various cell types, including chondrocytes, have been shown interact with FN (Hook 1984, Barkalow 1994). It was not determined whether NG2/HMPG could modulate integrin interaction with FN without CS chains (Iida 1995).

To determine the biochemical characteristics of NG2/HMPG in cartilage, and to compare them to that of NG2/HMPG in rat cell lines and human melanoma cells, western blotting of lysates of chondrocytes was carried out. NG2/HMPG is expressed simultaneously as both the CS rich PG and the unmodified protein core in



human chondrocytes isolated from both normal and OA cartilage, as in rat and melanoma cell lines.

The function of the two forms of NG2/HMPG is unclear, expression as both a CSPG and a CS-free protein core may act as a signal for transport to cell surface, or a regulatory mechanism. CD44 is expressed as both a PG and unmodified core protein. This integral membrane protein has a tissue specific variation in expression. In lymphocytes the majority of CD44 is expressed as unmodified protein core, but in epithelial cells core protein amino acid composition differs and the majority of CD44 is expressed as CS/HSPG. CD44 interacts with FN, collagen and HA. However, not all CD44-expressing cells bind HA, and different CD44-expressing cell types bind HA with different affinities. Only the CS modified form of CD44 interacts with FN. The expression of this protein as different forms is a possible means of regulating function (Hardingham 1992). Syndecan is a PG which has a structure similar to NG2/HMPG, consisting of a short intracellular domain with a single transmembrane span and a large extracellular region. The GAG content of the molecule expressed is cell specific. Simple mouse uterine epithelia express syndecan with 2HS and 2CS attached. Stratified vaginal epithelia express syndecan with only 1HS and 1CS attached. Transforming growth factor- $\beta$  (TGF $\beta$ ) increases the CS content of syndecan expressed by mouse mammary cells. The addition of CS enables interaction with thrombospondin. Therefore, ligand specificity is encoded by the GAG attached, and can be modified by cytokines during development and differentiation (Hardingham 1992). Thrombomodulin is expressed as a CS-rich and CS-free PG, both of which have distinct functions, the former mediates anti-coagulation events, and the latter is involved in their regulation (Hardingham 1992).

NG2/HMPG is expressed as both a CSPG and a CS free core protein in human chondrocytes. Addition of CS may enable NG2/HMPG to interact with ligands, and the core protein may be responsible for regulating these interactions. In this way, two distinct functions can be performed by different forms of one protein.

### **4.3. DIRECT INTERACTION OF NG2/HMPG WITH ECM IN HUMAN CHONDROCYTES**

Putative roles for NG2/HMPG in the binding of CII and CVI and in modulating melanoma cell interactions with FN have been proposed (Burg 1996, Iida 1995). These are particularly relevant to chondrocytes, as both CVI and FN are expressed in the pericellular matrix of cartilage (Clemmensen 1982, Poole 1992), and CII is the main collagen component of the ECM (Kuhn 1987). Human melanoma cells adhere to FN, laminin, CIV or anti-HMPG antibody 9.2.27 without cell spreading and focal contact formation (Iida 1995). However, when cells are allowed to adhere to a composite of both FN and 9.2.27, cell spreading and focal contact formation is observed (Iida 1995), suggesting that NG2/HMPG acts as a co-receptor for FN, and is involved in the control of melanoma cell spreading and focal contact formation. Studies with rat cell lines have suggested roles in binding to CVI. Immunoprecipitation of NG2/HMPG with anti-NG2 antibody from some rat cell lines including B49, but not others, results in a 140 kD component being co-precipitated along with the CSPG and unmodified core protein of NG2/HMPG (Stallcup 1990). This was subsequently identified as CVI, suggesting an interaction between the two (Stallcup 1990). Further studies have shown that CVI and NG2/HMPG co-localise on the surface of these cells (Stallcup 1990). In cell lines that secrete, but do not retain CVI, transfection with NG2/HMPG cDNA and subsequent expression of NG2/HMPG enables these cells to retain CVI on the cell surface (Nishiyama 1993). Adhesion assays using purified proteins have further characterised this interaction, and shown that it can be inhibited by decorin, indicating that they bind to the same, or closely related domains in CVI (Burg 1996). These binding studies also showed that NG2/HMPG interacts with CII (Burg 1996).

This evidence prompted an examination of whether NG2/HMPG interacts with these molecules in human cartilage. Immunofluorescence staining revealed that NG2/HMPG is distributed in a punctate pattern on chondrocytes isolated from normal and OA cartilage, identical to that seen at the cell surface of rat cell lines and human melanoma cells. Immunoprecipitation and double immunofluorescence

labelling of chondrocytes isolated from normal cartilage suggests that no direct interaction occurs with FN, CII or CVI.

In this study, only the unmodified core protein was precipitated from radiolabelled chondrocyte extracts. Rettig (1986) used M28B5 antibody to precipitate NG2/HMPG from human and mouse hybrid neuroblastoma cells, and noted that the 250 kD glycoprotein was the major component of the immunoprecipitate, and that the CSPG was detectable but relatively minor. Other groups, using different antibodies, report an absence of CSPG due to ineffective labelling or transfer to nitro-cellulose as a result of its large size or culture conditions of the melanoma cells from which it was isolated (Carrel 1981, Morgan 1981). The CSPG could only be isolated from large amounts of protein source, and detected as a minor band after labelling with radio iodine and extended exposure to x-ray film (Wilson 1981), and was not detected by Coomassie Blue staining of melanoma ghost preparations (Ross 1983).

In contrast to B49 rat cell lines, NG2/HMPG in human chondrocytes does not appear to directly interact with CVI. However, in other rat cell lines no co-precipitation or co-localisation was shown (Stallcup 1990), indicating that NG2/HMPG's role may be cell as well as tissue specific. NG2/HMPG also does not appear to interact with CII or FN. However this does not rule out a functional partnership, as has been demonstrated for FN in human melanoma cells (Iida 1995).

#### **4.4. A ROLE FOR NG2/HMPG IN MEDIATING CHONDROCYTE ADHESION TO CVI**

This thesis presents no evidence that NG2/HMPG interacts directly with any ECM protein tested. NG2/HMPG in human melanoma cells is not localised to focal contacts, and no direct interaction with FN has been demonstrated (Iida 1995). Instead, NG2/HMPG influences melanoma cell interaction with FN indirectly, by modulating  $\beta 1$  integrin binding of this ECM protein (Iida 1995). Normal human chondrocytes express integrins  $\alpha 1\beta 1$ ,  $\alpha 3\beta 1$ ,  $\alpha 5\beta 1$  and  $\alpha V\beta 5$  (Salter 1992, Loeser 1997, Woods 1994). Integrins  $\alpha 1\beta 1$  and  $\alpha 3\beta 1$  are involved in the binding of human chondrocytes to CII, and  $\alpha 3\beta 1$ ,  $\alpha 5\beta 1$  and  $\alpha V\beta 5$  to FN (Loeser 1995, Enomoto

1993). Human chondrocyte attachment to CVI is less well characterised, but is thought to occur by interaction with  $\alpha 1\beta 1$  integrins (Salter unpublished observations), which is supported by studies with bovine chondrocytes (Loeser 1997) and other cell types (Pfaff 1993).

Adhesion assays were used to characterise chondrocyte adhesion to ECM proteins FN, CII and CVI, and to study the effect of anti-HMPG and anti- $\beta 1$  integrin antibodies on this process. Chondrocytes isolated from normal and OA cartilage adhere to FN, CII and CVI. Binding to BSA is minimal and non specific. Anti- $\beta 1$  integrin antibody P4C10 inhibits the adhesion to FN, CII and CVI of chondrocytes isolated from normal and OA cartilage, confirming that  $\beta 1$  integrins are involved in mediating chondrocyte adhesion to all three substrates. Anti-HMPG antibody M28B5 and anti- $\beta 1$  integrin antibody TS2/16 have little effect on adhesion of chondrocytes isolated from normal cartilage to FN and CII, and adhesion of chondrocytes isolated from OA cartilage to FN, CII and CVI. However, they do significantly stimulate the adhesion of chondrocytes isolated from normal cartilage to CVI, in a dose dependent manner.

NG2/HMPG may have a role in influencing the interaction of chondrocytes from normal cartilage to CVI. This is altered in chondrocytes isolated from OA cartilage allowed to adhere to CVI, and in both cell types when allowed to adhere to FN or CII. Cell adhesion molecules, for example  $\beta 1$  integrin and CD44, not only mediate chondrocyte adhesion to ECM proteins but also regulate many other cell functions. Similarly NG2/HMPG appears to be important in modulating chondrocyte adhesion to CVI, and this may have implications for NG2/HMPG's role in a wide variety of chondrocyte functions, including regulation of matrix synthesis and degradation and proliferation. The expression of cell adhesion molecules is altered in OA cartilage, for example an upregulation in both  $\beta 1$  integrin and CD44 occurs. As both these molecules are involved in mediating cell-ECM interactions, which regulate chondrocyte function in normal cartilage, changes in expression suggests an alteration of these processes in OA cartilage. NG2/HMPG expression is also altered in OA cartilage, raising the possibility that the chondrocyte-ECM interactions it

modulates are altered which may affect subsequent regulation of cellular function and this may play a role in the onset and progression of osteoarthritis.

CVI interacts with several cartilage ECM proteins, for example CII and decorin (Bidanset 1990, Burrows 1994). Its localisation in the pericellular matrix also enables it to interact with cell surface receptors (Poole 1992). Chondrocyte interactions with CVI are important in the organisation of the ECM, to maintain tissue stability, provide structural support for the cells, and in the determination of cell morphology. However, the ECM also modulates cell behaviour by interacting with cell surface receptors and transmitting signals from the outside of the cell to the inside (Gimond 1992). Cell adhesion and spreading of smooth muscle and melanoma cells, is promoted by CVI and mediated by integrins (Aumailley 1989). CVI promotes migration in rat cell lines which express NG2/HMPG, but not in cell lines that do not express NG2/HMPG (Burg 1997), and melanoma cells that express CVI are more invasive than those that do not (Daniels 1996). CVI stimulates proliferation of transformed mesenchymal cells *in vitro* at a site different from that used to adhere to the cells via  $\beta 1$  integrins (Atkinson 1996). In these cells, NG2/HMPG is a candidate for mediating the proliferative effect of CVI. NG2/HMPG may be important in controlling normal chondrocyte adhesion to CVI, and therefore in the maintenance of cartilage structure and function.

NG2/HMPG does not appear to mediate chondrocyte attachment to CVI directly, but may do so by modulating  $\beta 1$  integrin interaction with CVI, as occurs with NG2/HMPG and FN in human melanoma cells (Iida 1995). The work presented in this thesis confirms that  $\beta 1$  integrins do mediate chondrocyte adhesion to CVI, as well as to FN and CII. Cellular recognition of ECM proteins involves the co-ordinated action of several distinct receptors. For example, fibroblasts require both the RGD-containing and heparin binding domains of FN to adhere to, and form focal contacts on FN, suggesting that both integrins and GAGs are involved (Guan 1991). Chinese hamster ovary cells, deficient in GAGs, adherent to FN form fewer focal contacts (Lebaron 1988). In human melanoma cells both CSPG and  $\alpha 4\beta 1$  integrin are involved in the interaction with FN (Iida 1992). Stimulation of both  $\alpha 4\beta 1$  and NG2/HMPG are required for cell spreading and focal contact formation on FN,



however they do not have to be localised on the same substrate to collaborate (Iida 1995). Presentation of antibody 9.2.27 on beads to cells already adherent on FN causes the same stimulation of spreading and focal contact formation as allowing cells to adhere to a composite substrate of FN and 9.2.27. The same is not true for FN presented on beads to cells adherent to 9.2.27. This suggests that  $\alpha 4 \beta 1$  must be in contact with the FN substrate, but that NG2/HMPG need not, and modulates the interaction of  $\alpha 4 \beta 1$  integrin via inside out signalling (Iida 1995). In other cell types integrin affinity for ECM ligands is regulated by inside out pathways involving intracellular signalling cascades, leading to a change in the conformation of the integrin (Hynes 1992). The mechanisms by which this change in conformation modulates ligand interaction are not well established.

Stimulation of chondrocytes, isolated from OA cartilage, with anti-HMPG antibodies does not result in the same increase of adhesion to CVI, suggesting that NG2/HMPG is not involved in mediating adhesion to CVI in the same way in chondrocytes isolated from OA cartilage as those isolated from normal cartilage. OA cells produce less NG2/HMPG and more CVI and  $\beta 1$  integrin (Swoboda 1998, Loeser 1995). This increase in  $\beta 1$  and CVI may be a reparative event to remodel cartilage, to balance degradation by proteases.  $\beta 1$  integrin and FN production are upregulated in OA cartilage, and mediate cell-ECM interactions during cartilage repair (Clancy 1997). Degradation of NG2/HMPG, which in normal cartilage promotes interaction between  $\beta 1$  and CVI, may cause an increase in the production of these molecules to compensate for loss of stimulatory effect. Alternatively, NG2/HMPG stimulation of cell-CVI interaction may only be needed in normal cells when lower amounts of CVI are present, and when levels of these proteins are upregulated, a parallel down regulation in NG2/HMPG expression may occur.

However, the small number of samples used in this study, the wide margin of error of adhesion values, and the lack of effect of TS2/16 on adhesion to CII and FN, suggests that a larger number of experiments may reveal that treatment of both normal and OA cells with M28B5 may stimulate binding to CVI or CII and FN. NG2/HMPG may not specifically modulate the adhesion of chondrocytes isolated from normal cartilage to CVI, but influence both the adhesion of chondrocytes

isolated from normal and OA cartilage to a wider range of substrates. This needs to be examined using a greater number of tests.

NG2/HMPG may modulate  $\beta 1$  integrin interaction with CVI which, potentially regulates normal cartilage structure and function, and which is altered in OA cartilage.

#### **4.5. NG2/HMPG IN CHONDROCYTE SIGNAL TRANSDUCTION**

How does NG2/HMPG modulate chondrocyte adhesion to CVI? With regards to a signalling molecule, NG2/HMPG appears to have many potential roles. As a transmembrane molecule it can influence events on both sides of the membrane. NG2/HMPG has been shown to stimulate the activity of tyrosine specific kinases in human melanoma cells (Iida 1995), to act as a cytoskeletal anchor by interacting with actin in rat cell lines (Lin 1996a), to mediate optimal cell response to PDGF by interacting with PDGF $\alpha$ -R in rat O-2A and smooth muscle cell lines (Nishiyama 1996a,b, Grako 1995), to activate Gi proteins by binding to a cell surface receptor on rat neurites (Dou 1994), and it may also be a substrate for PKC (Nishiyama 1995).

To further dissect how NG2/HMPG can regulate chondrocyte interaction with CVI; chondrocytes isolated from normal cartilage to CVI were used in adhesion assays in the presence of inhibitors of intracellular signalling molecules.

##### **4.5.1. G protein mediated signalling**

Many cellular responses stimulated by extracellular signals use guanosine triphosphate (GTP) binding regulatory proteins (G proteins) to transduce the message into the cell. These signalling molecules are found on the intracellular face of the cell membrane and commonly function as receptors for hormones. Pertussis toxin sensitive Gi proteins regulate the activity of a wide variety of effector molecules, including adenylate cyclase, calcium, potassium and sodium ion channels and phospholipase enzymes, and intracellular concentrations of cyclic guanine monophosphate (cGMP), inositol triphosphate and diacyl glycerol (DAG) (Hepler 1992, Neer 1995).



Pertussis toxin, which inhibits the activity of Gi proteins, has no effect on chondrocyte adhesion to CVI in the absence of anti-HMPG antibody M28B5, however it inhibits the adhesion to CVI stimulated by the antibody. This indicates that chondrocyte adhesion to CVI in the absence of M28B5 does not involve pertussis toxin sensitive G proteins, however the stimulated adhesion of chondrocytes to CVI does involve Gi protein mediated signalling.

This thesis proposes that NG2/HMPG mediates the interaction of human chondrocytes isolated from normal cartilage with CVI, not via direct binding but indirectly by modulating  $\beta 1$  integrin interaction with CVI. Integrin affinity for ligands is regulated in some cells by G proteins. Stimulation of muscarinic receptors which are coupled to G proteins, results in DAG activation of PKC, which causes an increase in the affinity of integrins for the ECM, the number of stress fibres and focal contacts formed, and the tyrosine phosphorylation of paxillin and focal adhesion kinase (FAK) (Slack 1998). Integrin mediated adhesion of fibroblasts to FN involves Gi protein activation (Sung 1996). Interaction of  $\alpha II\beta 3$  integrin with fibrinogen in lymphocytes can be activated by pathways involving Gi proteins (Qi 1998). Gi proteins are more abundantly expressed in highly metastatic melanoma cells compared to normal cell counterparts or poorly metastatic melanoma cells (Lester 1991). In rat neurites NG2/HMPG binding to a cell surface receptor inhibits cell growth. This involves activation of Gi proteins and ion channels, and changes in the levels of intracellular cAMP and calcium (Dou 1994).

There is little evidence concerning the signal transduction pathways involving CVI, and none to suggest that Gi proteins are involved. However, the effect of some ECM proteins on Gi protein-mediated signal transduction has been studied. Tumour necrosis factor- $\alpha$  inhibits expression of the collagen I gene via G protein-mediated signalling (Hernandez-Munro 1997). FN interaction with integrins on rat neurite cells causes the activation of Gi proteins which alter the activity of potassium channels resulting in cell hyperpolarisation and increased cell growth (Arcangeli 1993). Pertussis sensitive G proteins and integrin  $\alpha V\beta 3$  are involved in human melanoma cell migration on vitronectin (Aznavorian 1996), and  $\alpha 4\beta 1$  adhesiveness for FN is decreased by chemokines, which is mediated by Gi proteins (Weber 1996).

No work has been done on CVI-induced signalling in human chondrocytes. This thesis is the first to report that NG2/HMPG may be involved in modulating chondrocyte interaction with CVI. The function of NG2/HMPG in human adult articular chondrocytes isolated from normal cartilage may be to stimulate cell adhesion to CVI by Gi protein mediated signalling, possibly by modulating  $\beta 1$  integrin interaction with this ECM protein.

Evidence exists for G protein regulation of chondrocyte function. In chondrocytes from species other than humans, TGF- $\beta$  decreases adhesion to CVI by decreasing expression of its receptor  $\alpha 1 \beta 1$  (Loeser 1997). TGF- $\beta$  enhances chondrocyte differentiation via activation of Gi proteins (Schwartz 1988). Insulin like growth factor (IGF) interaction with IGF-1R and IR stimulates chondrocyte proliferation and ECM protein synthesis by activation of Gi proteins and stimulation of phospholipase C (PLC)/inositol triphosphate-mediated opening of endoplasmic reticular calcium ion channels to create an increase in intracellular calcium concentration (Poiraudau 1997). Mechanical force results in a change in shape of bovine chondrocytes, an increase in GAG and metalloproteinase synthesis and eventual degradation of cartilage. The increase in GAG synthesis involves nitric oxide release and Gi protein activation of PLC, by DAG generation, and may activate PKC which has been shown to increase GAG synthesis in cultured chondrocytes (Das 1997). Gi proteins therefore are involved in the control of cartilage metabolism in the degenerative disease of this tissue. In human chondrocytes NG2/HMPG may use Gi proteins to control cell adhesion to CVI, which in OA cartilage may be involved in initial up regulation of PG synthesis. The question remains as to how NG2/HMPG modulation of chondrocyte interaction with CVI is regulated.

#### **4.5.2. Phosphorylation of NG2/HMPG by PKC**

NG2/HMPG was sequenced and found to show little homology to any other protein yet characterised (Nishiyama 1991b, Pluschke 1996). The internal domain of NG2/HMPG contains three threonine residues (Nishiyama 1991b, Pluschke 1996) which, together with surrounding amino acids, form a motif for potential PKC phosphorylation (Kemp 1990), indicating that NG2/HMPG may be modified on these

amino acids and so play a role in intracellular signalling. Western blots of chondrocyte lysates probed with anti-phosphothreonine antibodies reveal a wide range of proteins which are phosphorylated on threonine, including one which migrates at 250 kD, suggesting that NG2/HMPG may be a substrate for PKC.

Further work is needed to confirm that NG2/HMPG is phosphorylated by PKC. The PKC phosphorylation site is one of very few sequences present in NG2/HMPG that is highly conserved in both the rat and human molecule and other proteins (Nishiyama 1991b, Pluschke 1996). In rat cell lines, activation of PKC stimulates the production of a truncated form of NG2/HMPG and an inability to anchor CVI. PKC may directly phosphorylate NG2/HMPG, causing a change in conformation and a decrease in affinity for CVI, without which interaction NG2/HMPG is not protected from proteolytic enzymes, a property conferred by the protease inhibitor sequences of CVI (Nishiyama 1995). Alternatively, PKC activation may result in the synthesis of a protease responsible for the truncation of NG2/HMPG which renders it incapable of interaction with CVI (Nishiyama 1995). In this way PKC may act as a means of regulating the direct interaction of NG2/HMPG and CVI. However, NG2/HMPG does not directly interact with CVI in human chondrocytes. PKC may regulate the direct interaction of NG2/HMPG with a novel or unidentified ligand.

Alternatively PKC may mediate other chondrocyte functions. The adhesion assay results show that inhibition of calcium dependent PKC has no effect on adhesion to CVI of chondrocytes isolated from normal areas of knee joints either in the absence or presence of stimulating anti-HMPG antibody M28B5. This suggests that PKC is not involved in the adhesion of chondrocytes to CVI in the absence of antibody, or when stimulated by anti-HMPG antibody M28B5.

In some cell types, including chondrocytes, PKC is not involved in the adhesion of cells to ligands, but in cellular functions that occur after this, for example cell spreading, focal contact formation and migration. In B and T cells, integrin-ligand interaction promotes cell adhesion, but direct activation of PKC or additional receptor ligand interaction is required for focal contact formation (Woods 1992). Platelet activation involves two steps, PKC independent, GTP regulatory protein

dependent (Daniel 1992) attachment of platelets to collagen by  $\alpha 2\beta 1$  integrins, followed by platelet aggregation and secretion, for which PKC is required (Karniguan 1990). Similarly, integrin interaction with the RGD domain of FN is sufficient for human fibroblast adhesion and spreading, however the heparin binding domain and PKC activation is required for focal contact formation (Woods 1992). The additional signals needed for focal contact formation are provided by the HSPG syndecan, which is localised to focal contacts and is thought to act as a co-receptor which modulates  $\beta 1$  integrin interaction with FN (Woods 1994). Human melanoma cells are able to adhere to FN, they require stimulation of NG2/HMPG to spread and form focal contacts (Iida 1995). A similar mechanism may occur in chondrocytes; NG2/HMPG stimulation of adhesion of integrins to CVI via a Gi protein dependent, PKC independent pathway, followed by PKC dependent spreading and focal contact formation.

#### **4.5.3. Stimulation of tyrosine kinase activity by NG2/HMPG**

Human melanoma cells are able to adhere to FN, but they require stimulation of NG2/HMPG to spread and form focal contacts (Iida 1995). During the stimulated cell spreading and focal contact formation two proteins of molecular weight 120 and 130 kD are tyrosine phosphorylated. This can be inhibited by tyrosine kinase inhibitors (Iida 1995). There is little information about tyrosine phosphorylation of specific proteins in chondrocytes. Mechanical strain promotes the phosphorylation of FAK and paxillin (Lee 1998). Lipocortin tyrosine phosphorylation is upregulated by inhibition of protein tyrosine phosphatases (Grima 1994), and MAP kinases ERK 1 and 2 phosphorylation is stimulated by IL-1, PDGF and IGF-1 (Geng 1996).

Preliminary comparison of the profile of proteins tyrosine phosphorylated in human chondrocytes isolated from normal cartilage and plated on BSA and those stimulated by allowing them to adhere to FN, CVI, M28B5, KZ1, TS2/16 and mixtures of FN and M28B5 antibody, showed no difference. However chondrocytes were only allowed to adhere to substrates for 1 h, with the exception of M28B5, where cells were plated on a time course ranging from 1 min to 1 h. This length of time is sufficient for chondrocytes to adhere, however after 1 h the chondrocytes are

still rounded and have not begun to spread, suggesting that tyrosine kinase activity is not required for the initial attachment to substrates. Further work is needed to study the tyrosine phosphorylation events in chondrocytes which have been allowed to adhere for longer periods of time, and spread and form focal contacts.

The adhesion assay results showed that inhibition of tyrosine specific kinases has no effect on adhesion to CVI of chondrocytes isolated from normal cartilage either in the absence or presence of stimulating anti-HMPG antibody M28B5. This suggests that these kinases are not involved in the intracellular signals required for cellular interaction with CVI. Tyrosine kinases, like PKC may be involved in functions such as cell spreading, focal contact formation and migration.

Chondrocyte adhesion to FN, CI and CII is not affected by inhibitors of tyrosine kinases, however migration promoted by FN, types I and II collagen is blocked (Shimizu 1997). Expression of NG2/HMPG by rat cell lines correlates with the ability to migrate to CVI (Burg 1997) although the mechanism by which this occurs is not known. FN binding of  $\alpha 5 \beta 1$  integrin in chondrocytes is followed by integrin clustering and focal contact formation including actin, and tyrosine kinase FAK and Rho A recruitment (Clancy 1997). Nitric oxide inhibits focal contact formation but not integrin ligation or clustering (Clancy 1997), and nitric oxide release is dependent on tyrosine kinase activity (Geng 1995).

PKC and tyrosine specific kinases appear not to be involved in initial cell adhesion, but may play a role in cell spreading, focal contact formation or migration. In adenocarcinoma cells PKC activation correlates with FAK phosphorylation, cell spreading and focal contact formation, involving recruitment of actin and  $\beta 1$  integrins (Ghisso 1997). Crosslinking of  $\alpha 4 \beta 1$  in T cells induces migration on FN, laminin and CIV. This is inhibited by PKC and tyrosine kinase inhibitors. Crosslinking of CD3 triggers ligand binding and migration (Hauzenberger 1997).

Phosphorylation events play a role in the regulation of integrin-ligand affinity. In T cells, cross linking of the CD3 cell surface molecule results in activation of  $\alpha 4 \beta 2$  integrin and stronger adhesion to ligands via the activation of PKC (Hynes 1992). Src tyrosine phosphorylation of  $\beta 1$  integrin in transformed cells decreases  $\beta 1$  binding to talin and FN. Serine phosphorylation of  $\alpha 5 \beta 1$  integrin in mitotic cells



reduces binding to FN, and allows cells to round up and detach in preparation for cell division (Hynes 1992). Damage to the endothelial lining causes an increased adhesion of platelets to collagen via  $\alpha 2\beta 1$  integrins which stimulates tyrosine phosphorylation of PLC, resulting in its increased activity and platelet aggregation and secretion (Poole 1997). In the same way NG2/HMPG may facilitate integrin interaction of chondrocytes with CVI via Gi proteins, and trigger cell spreading, focal contact formation and migration involving PKC and tyrosine kinases.

#### **4.5.4. Interaction of NG2/HMPG with actin**

NG2/HMPG expression in flattened rat cell lines is found to be in ordered linear arrays which co-localise with the cytoskeletal stress fibres of actin, but not microtubules or intermediate filaments (Lin 1996a). Cytochalasin D, which disrupts actin polymerisation, destroys both patterns of expression and causes NG2/HMPG to be released from the cell. In cells which have rounded up in preparation for migration or a change in adhesive environment, the two no longer co-localise, suggesting that the co-localisation is specific, and functions in the regulation of cell adhesion and spreading (Lin 1996a). Human adult articular chondrocytes in culture do not form stress fibres, but actin is distributed in spindle like arrays, which radiate out from the centre of the cells to the edges (Wright 1997).

Disruption of the cytoskeleton with cytochalasin D results in a dramatic decrease in chondrocyte ability to interact with CVI, both with and without M28B5. This is unsurprising, as the actin cytoskeleton acts as an anchor for  $\beta 1$  integrins in focal contacts, which are involved in CVI adhesion. From these studies, it is impossible to determine whether this agent is inhibiting general chondrocyte adhesion to CVI, or M28B5 stimulated chondrocyte adhesion to CVI.

Actin is not clearly co-localised with NG2/HMPG, which is expressed in a punctate pattern, suggesting that, in human chondrocytes, at least *in vitro*, NG2/HMPG does not appear to be involved in the anchoring of the cytoskeleton and subsequent regulation of cell adhesion, as in some rat cell lines. The actin cytoskeleton is required for chondrocyte interaction with CVI, however the lack of co-localisation in chondrocytes with NG2/HMPG suggests that it does not do so via

an interaction with NG2/HMPG. It exerts its effect by interacting with other molecules such as integrins, and NG2/HMPG may act by interacting with other intracellular proteins.

#### **4.5.5. Interaction of NG2/HMPG with PDGF $\alpha$ -R**

In rat O-2A cells *in vitro* and *in vivo*, NG2/HMPG and PDGF $\alpha$ -R expression is closely co-localised and co-regulated throughout development, until maturation in the postnatal/adult rat (Nishiyama 1996a). *In vitro* co-expression and interaction of the two molecules corresponds with cell ability to respond optimally to PDGF (Nishiyama 1996b). In rat smooth muscle cell cultures, NG2/HMPG and PDGF $\alpha$ -R are also co-expressed, and anti-NG2 antibodies interfere with the ability of cells to respond to PDGF, implicating NG2/HMPG in the regulation of PDGF and PDGF $\alpha$ -R interaction (Grako 1995). Normal human adult articular chondrocytes in culture do not proliferate, but are slow growing cells (Caterson 1997). PDGF $\alpha$ -R is not clearly co-localised with NG2/HMPG, which is expressed in a punctate pattern, suggesting that in human chondrocytes, at least *in vitro*, NG2/HMPG does not appear to be involved in the regulation of PDGF and PDGF $\alpha$ -R interaction and subsequent modulation of cell proliferation as in rat O-2A and smooth muscle cell lines.



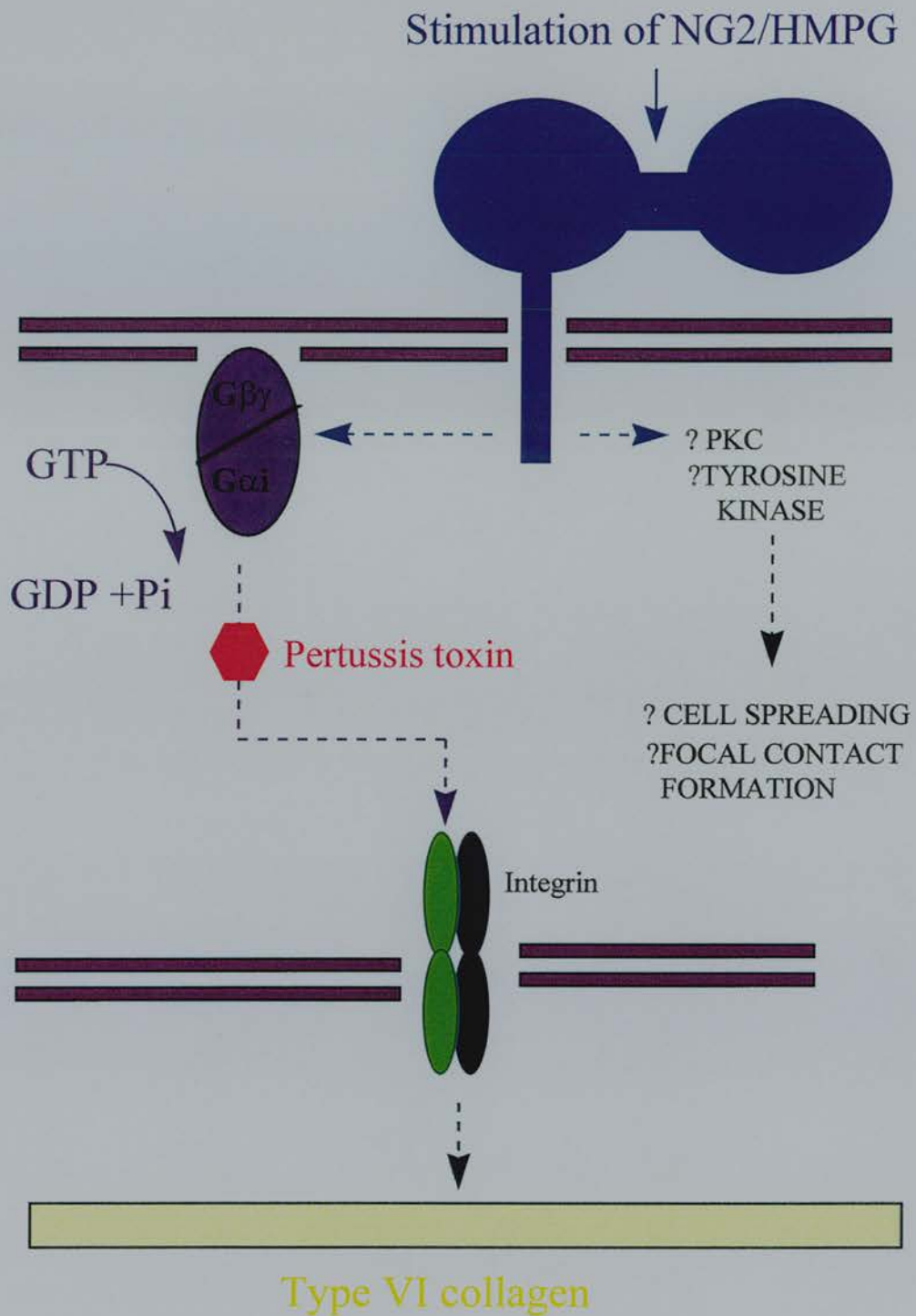
## 4.6. CONCLUSIONS

The results of this study show that the novel cell membrane associated CSPG NG2/HMPG is expressed by chondrocytes in human adult and fetal cartilage. Currently the role of NG2/HMPG in chondrocytes remains unclear. Work with rat cell lines and melanoma cells suggest that NG2/HMPG function may be cell type specific (Stallcup 1990, Iida 1995). NG2/HMPG modulates the adhesion to CVI of chondrocytes isolated from normal cartilage. NG2/HMPG does not directly interact with any protein so far tested, therefore it may indirectly influence this adhesion. Indeed, previous work suggests that articular chondrocytes use integrins predominantly in their adhesive interactions with these molecules (Loeser 1995, 1997, Enomoto 1993, Durr 1993). This raises the possibility that NG2/HMPG regulates chondrocyte-ECM interaction indirectly via modulation of integrin affinity for its ligands. This modulation of chondrocyte adhesion to CVI requires the activation of G $\alpha$  proteins, which may directly, or through the activation of one or more effector molecules, alter the affinity of  $\beta$ 1 integrin for CVI.

NG2/HMPG may also be involved in signalling pathways which activate PKC and tyrosine specific kinases, but which are not involved in cell adhesion to CVI. They may mediate alternative cellular functions, such as migration, spreading and focal contact formation. This is important *in vitro*, however is of limited relevance *in vivo*, where chondrocytes do not spread or migrate.

This theory proposes two separate functions for NG2/HMPG. NG2/HMPG potentially participates in two or more distinct signalling pathways, in the regulation of cell adhesion to CVI via G protein-mediated control of integrin affinity, in the activation of tyrosine kinases, and as a substrate for PKC which may be involved in other cell functions such as migration, spreading and focal contact formation (FIGURE 4.1.). The expression of NG2/HMPG as both unmodified protein core and CSPG may be a means of regulating multiple functions, for example, which signalling pathway occurs, and ligand affinity and specificity.

NG2/HMPG expression is down regulated in OA cartilage. Stimulation of chondrocytes from OA cartilage with anti-HMPG antibodies does not increase adhesion to CVI, suggesting that normal function is altered in the disease process.



**FIGURE 4.1.**

Proposed role of NG2/HMPG in normal human articular chondrocytes. NG2/HMPG indirectly stimulates chondrocyte adhesion, by modulating integrin affinity for CVI via inside out signalling pathways involving Gi proteins. NG2/HMPG may also be involved in chondrocyte spreading and focal contact formation.

## 4.7. FUTURE WORK

### **The function of NG2/HMPG in chondrocytes isolated from normal cartilage**

*The role of NG2/HMPG in adhesion to the ECM.* This thesis has provided evidence that NG2/HMPG does not directly interact with FN, CII or CVI, however it may directly bind to other proteins found in the ECM of cartilage. Direct binding assays have shown NG2/HMPG isolated from rat cell lines to interact with other proteins found in cartilage ECM (Tillet 1997). Other candidate ligands should be explored, for example laminin and tenascin, including the possibility that NG2/HMPG directly binds a novel ligand. Future work should also include determination of any direct interaction of NG2/HMPG with ECM proteins after removal of the CS GAGS.

*Gi protein mediated signal transduction.* In human chondrocytes NG2/HMPG may use Gi proteins to control cell adhesion to CVI. The question remains as to how NG2/HMPG modulation of chondrocyte interaction with CVI is regulated and how integrins are involved. The up/downstream events, such as how stimulation of NG2/HMPG occurs and how the activation of Gi proteins leads to an increase in the adhesion of chondrocytes to CVI, should be investigated. For example, does NG2/HMPG interact directly with the G protein, or via an extra loop involving other membrane protein receptors or secreted proteins, and how does this affect adhesion and regulate integrin function? Potential intracellular second messengers include adenylate cyclase, PLC, inositol triphosphate, DAG, PKC, ion channel regulation and decreased cGMP concentrations. The effect of inhibitors of downstream molecules, and any changes in intracellular molecule concentration, for example calcium, cAMP, DAG, inositol triphosphate and arachidonic acid should be studied. Alternatively, NG2/HMPG may signal via a novel intracellular pathway.

*The role of NG2/HMPG in chondrocyte spreading and focal contact formation.*

Chondrocytes were stimulated with antibody or ECM proteins, and changes in tyrosine phosphorylation looked for. However, cells were only allowed to adhere to a limited set of substrates for a short period of time. Cells must be allowed to adhere

to, and spread on, anti-HMPG antibodies, FN, CVI and composite substrates. Chondrocyte focal contact formation should be also studied. It will be important to extend the time course to enable events beyond adhesion to be analysed.

NG2/HMPG's primary sequence suggests that it is a substrate for PKC. Activation of PKC in rat cells stimulates the appearance and secretion of a truncated NG2/HMPG, and a decrease in interaction with CVI. The effect of PKC activation on NG2/HMPG expression, size, retention at the cell surface, and ability to stimulate adhesion to CVI should be looked at. It will also be important to determine whether NG2/HMPG is a substrate for phosphorylation.

The participation of NG2/HMPG in signalling pathways which may involve growth factor stimulation suggests that studying the effect of growth factors including TGF- $\beta$ , PDGF or cytokines such as IL-1 on NG2/HMPG expression may be of interest. Understanding of what regulates NG2/HMPG expression may provide insight into the function of NG2/HMPG in both normal cartilage and in the OA disease process, as well as the starting point for the stimulated adhesion to CVI.

*Other intracellular functions.* This work has shown no co-localisation of NG2/HMPG with actin in chondrocytes isolated from normal cartilage; however, NG2/HMPG may have a different function in chondrocytes isolated from OA cartilage. In cell types other than rat cell lines, potential intracellular ligands have not been studied. Actin does not comprise the whole cytoskeleton, for example, in focal contacts integrins are connected to actin stress fibres by an indirect linkage via talin, vinculin, paxillin,  $\alpha$ -actinin and capping protein. Microtubules, intermediate filaments, and actin accessory proteins for example filamin and myosin, also form part of the intracellular network. There are antibodies available which recognise these proteins, by which NG2/HMPG may transduce signals between the outside and inside of the cell.

#### **The function of NG2/HMPG in chondrocytes isolated from OA cartilage**

It will be important to determine why expression of NG2/HMPG is down regulated in OA cartilage, and if this contributes to the disease process. The function of

NG2/HMPG should be investigated in chondrocytes derived from OA cartilage, including a comparison of the localisation of NG2/HMPG with ECM proteins and actin, and repetition of the adhesion assays to FN, CII and CVI in the presence of M28B5. NG2/HMPG in OA cartilage may modulate chondrocyte adhesion to different substrates, using different intracellular signalling pathways from those used in normal cartilage.

### **The function of the unmodified core and the CSPG**

It will be important to identify which form of NG2/HMPG, the unmodified protein core or the CSPG, is functionally active and if expression is a means of regulating function. Quantitation of the different forms of NG2/HMPG expressed in normal and OA cartilage would provide an insight into the function in the different cell types, as would the effect of chondroitinase digestion on the function of NG2/HMPG.

### **Molecular analysis**

NG2/HMPG function appears to be cell type specific. Determination of the DNA and AA sequence of NG2/HMPG from chondrocytes isolated from both normal and OA cartilage would allow sequence homology to rat NG2 and HMPG to be compared. Determination of cell specific variation in DNA or protein may account for different function. A comparison of the positions of exons/introns of NG2/HMPG, and of mRNA expressed in human chondrocytes to those seen in other species, may reveal splice variation that could account for the functional differences. The production and expression of fragments and mutant forms of NG2/HMPG may also provide an insight into the precise functional mechanisms of this protein.

It will be important to establish how NG2/HMPG is involved in the regulation of integrin function and identify potential ligand(s) in cartilage, to investigate possible functions of this molecule in cell signalling and regulation of chondrocyte activity in both normal cartilage and in diseases such as osteoarthritis where expression is modified.



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## APPENDIX I

Monoclonal antibodies used throughout this study.

ANTIBODY	CLONE	TYPE	SOURCE
Anti-rat NG2	N143.8	mouse monoclonal	William Stallcup, La Jolla Cancer Research Foundation, California USA
Anti-human HMPG	9.2.27	mouse monoclonal	William Stallcup, La Jolla Cancer Research Foundation, California USA
Anti-human HMPG	M28B5	mouse monoclonal IgG2A	American Tissue Culture Collection
Anti-CD44	KZ1	mouse monoclonal	David Anstee, International Blood Group Reference Laboratory, Bristol UK
Anti-FN	IST-4	mouse monoclonal	Luciano Zardi, Milan
Anti-phosphothreonine	PTR-8	mouse monoclonal	Sigma, Poole UK
Anti- $\beta$ 1 integrin	TS2/16	mouse monoclonal	Francisco Sanchez-Madrid, Madrid
Anti- $\beta$ 1 integrin	P4C10	mouse monoclonal IgG1	GIBCO, Life Technologies, Paisley UK
Anti-CD3	UCHT-1	mouse monoclonal IgG1	Seward, UK
Anti-phosphotyrosine	PT-66	mouse monoclonal	Sigma, Paisley UK
HRP-conjugated anti-phosphotyrosine	PY-20	mouse monoclonal	Transduction laboratories, Exeter UK

Polyclonal antibodies used throughout this study.

ANTIBODY	TYPE	SOURCE
Biotinylated anti-mouse Ig	rabbit polyclonal	DAKO, BUCKS UK
FITC conjugated anti-mouse Ig	rabbit polyclonal	DAKO BUCKS UK
Anti-PDGF $\alpha$ -R	rabbit polyclonal	Santa Cruz, USA
Anti-FN	rabbit polyclonal	Sigma, Poole UK
Anti-CII	rabbit polyclonal	Southern, Bionuclear Services, Reading UK
Anti-CVI	rabbit polyclonal	Southern, Bionuclear Services, Reading UK
FITC/TRITC conjugated anti-mouse Ig	goat polyclonal	Harlan labs, Loughborough UK
FITC/TRITC conjugated anti-rabbit Ig	goat polyclonal	Harlan labs, Loughborough UK
Anti-mouse Ig	rabbit polyclonal	Sigma, Poole UK



## APPENDIX II

Solutions used throughout this study.

### Solutions used in immunohistology

#### **10X TBS**

To 500 ml of 0.5 M Tris add approximately 200 ml 1 N HCl until pH is 7.6, make up to 2000 ml with distilled water and re-adjust pH to 7.6. For use dilute 1:10 with normal saline.

Normal saline	42.50 g sodium chloride
	5000 ml distilled water

#### **AB Buffer**

To 12 ml 0.2 M Tris add 19 ml 0.1 N HCl and 19 ml distilled water.

#### **DAB Substrate Solution**

To 0.2 ml DAB add 4.8 ml DAB buffer and 0.1 ml hydrogen peroxide solution.

Hydrogen peroxide solution	0.2 ml distilled water
	0.1 ml 30 %w/w hydrogen peroxide

DAB buffer (pH 7.6)	0.2 M Tris
	0.1 N HCl
	19 ml distilled water
	0.01 M imidazole

#### **Scott's tap water substitute**

Potassium carbonate	2 g
Magnesium sulphate	20 g
Distilled water	1 l

### **Solutions used in isolation and culture of chondrocytes.**

#### **Collagenase**

Type H collagenase: 3 mg per sample made up in 10 ml of serum free HAMs F12 medium and filter sterilised.

#### **Anti microbial solution.**

To 100 ml PBS add 5 ml each of Penicillin/Streptomycin (10 mg/ml), L-Glutamine (200 mM) and Fungizone (250 µg/ml).

### **Solution used for chondroitinase digestion**

#### **Chondroitinase ABC**

Stock solution: 2 units lyophilised chondroitinase ABC dissolved in 1 ml PBS and stored at -20 °C. For use dilute with PBS.

### **Solutions used for cell lysis**

#### **Lysis buffer (pH 7.4)**

10 mM Tris

5 mM EDTA

1 % NP-40

1 mM phenyl methyl sulphonyl fluoride (PMSF)

10 ml dH<sub>2</sub>O

### Solutions used for Lowry determination of protein concentration

#### **Alkaline carbonate solution**

To 5 ml copper sulphate solution add 5 ml sodium potassium tartrate and 490 ml alkaline carbonate stock solution.

Copper sulphate solution	1 g $\text{CuSO}_4$ in 100 ml $\text{dH}_2\text{O}$
Sodium potassium tartrate	2 g NaKtartrate in 100 ml $\text{dH}_2\text{O}$
Alkaline carbonate stock solution	20 g $\text{Na HCO}_3$ , 4 g $\text{NaOH}$ in 1 l $\text{dH}_2\text{O}$

#### **Folin's reagent**

Dilute 10 ml stock solution with 10 ml  $\text{dH}_2\text{O}$ , store in dark.

#### **BSA Standards**

Dilute stock BSA with 0.1 N  $\text{NaOH}$  to give final concentrations.

Stock BSA	1 mg BSA
	1 ml distilled water

BSA standards for Lowry determination of protein concentration

STANDARD NUMBER	FINAL BSA CONC. ( $\mu\text{g/ml}$ ).	VOL. 1 mg/ml BSA ( $\mu\text{l}$ )	VOL. 0.1N $\text{NaOH}$ ( $\mu\text{l}$ )
S1	400	80	120
S2	200	40	160
S3	150	30	170
S4	100	20	180
S5	75	15	185
S6	50	10	190
S7	25	5	195
S8	0	0	200

## Solutions used for SDS PAGE

### Separating gel composition

FINAL ACRYLAMIDE CONC. (%)	STOCK 30 % ACRYLAMIDE (ml)	SEPARATING GEL BUFFER (ml)	dH <sub>2</sub> O (ml)	10 % APS (μl)	TEMED (μl)
3	3.7	9.25	24.3	200	20
5	6.2	9.25	21.8	200	20
20	25.0	9.25	3.0	200	20

Ammonium persulphate (APS) (Amersham UK) was freshly prepared using dH<sub>2</sub>O.

### Stacking gel composition

FINAL ACRYLAMIDE CONC. (%)	STOCK 30 % ACRYLAMIDE (ml)	STACKING GEL BUFFER (ml)	dH <sub>2</sub> O (ml)	10 % APS (μl)	TEMED (μl)
2.5	0.85	2.5	6.35	30	10
4.5	1.5	2.5	5.7	30	10

### Separating gel buffer

pH to 8.8 and store at room temperature.

1.5 M Tris

0.5 % SDS

500 ml distilled water

### Stacking gel buffer

pH to 6.8 and store at room temperature.

0.5 M Tris

0.5 % SDS

500 ml distilled water

**10x Stock electrode buffer**

0.5 M Tris

0.5 M Glycine

1 % SDS

1 l distilled water

**Sample buffer**

0.5 M Tris pH 6.8

3.8 ml dH<sub>2</sub>O

0.8 ml glycerol

1.6 ml 10 % SDS

0.8 ml 0.1 M dithiothreitol (DTT)

0.2 ml 0.05 % bromophenol blue

## **Solutions used for electrophoretic transfer of proteins to nitro-cellulose**

### **Transfer buffer**

pH should be 8.3 but do not adjust, make up fresh each time.

3.03 g Tris

14.4 g Glycine

200 ml methanol

800 ml distilled water

## **Solutions used for development of blot using alkaline phosphatase**

### **Blocking solution**

10 mM Tris pH 8.0

150 mM NaCl

2 % BSA

0.02 % Na azide

### **TBST**

10 mM Tris pH 8.0

150 mM NaCl

0.05 % Tween

### **AP Substrate solution**

0.1 M Tris pH 9.0

0.1M NaCl

5 mM MgCl<sub>2</sub>

66 µl/10 ml Stock NBT

33 µl/10 ml Stock BCIP

Stock NBT 50 mg/ml in 70 % Dimethyl formamide

Stock BCIP 50 mg/ml in 100 % Dimethyl formamide

### **Solutions used for assessment of protein tyrosine phosphorylation**

#### **2x Lysis buffer**

prepare immediately before use

1 ml 1M Tris-Cl pH 8.0

2.3 ml 3 M NaCl

10 ml 50 % (v/v) glycerol

5 ml 10 % (v/v) NP-40

5  $\mu$ l 50 mM p-nitrophenyl guanidinobenzoate

50  $\mu$ l 10 mg/ml aprotinin

50  $\mu$ l 10 ml/ml leupeptin

0.5 ml 0.1M Na orthovanadate

0.2 ml 0.5 M EDTA pH 8.0

H<sub>2</sub>O to 25 ml



### APPENDIX III

Tissues sources used throughout this study.

**Tissues immunohistologically stained with anti-NG2/HMPG antibodies**

TYPE OF ARTICULAR CARTILAGE	SOURCE	NUMBER	SEX	MEDIAN AGE	RANGE
Normal	patella, tibia, femur	1	F	67 YEARS	
Normal	patella, tibia, femur	7	M	71 YEARS	53 - 88 YEARS
OA	patella, tibia, femur	13	M	71 YEARS	53 - 88 YEARS
Fetal	articular, epiphyseal, growth plate	10		17 WEEKS	14 - 20 WEEKS

**Tissues used in immunohistology over a range of anti-HMPG antibody dilutions**

TYPE OF ARTICULAR CARTILAGE	SOURCE	NUMBER	SEX	MEDIAN AGE (YEARS)	RANGE (YEARS)
Normal	patella, tibia, femur	1	F	67	
Normal	patella, tibia, femur	10	M	71	53 - 88
OA (mild)	patella, tibia, femur	4	M	61	53 - 69
OA (moderate)	patella, tibia, femur	6	M	64	53 - 74
OA (severe)	patella, tibia, femur	3	M	88	

**Tissues used in western blotting to detect NG2/HMPG**

<b>TYPE OF ARTICULAR CARTILAGE</b>	<b>SOURCE</b>	<b>NUMBER</b>	<b>SEX</b>	<b>MEDIAN AGE (YEARS)</b>	<b>RANGE (YEARS)</b>
Normal	tibia, femur	3	F	71	66 - 75
Normal	tibia, femur	9	M	62	46 - 78
OA	tibia, femur	1	F	66	
OA	tibia, femur	4	M	64	50 - 78

**Tissues used to determine band intensities of NG2/HMPG and FN**

<b>TYPE OF ARTICULAR CARTILAGE</b>	<b>SOURCE</b>	<b>NUMBER</b>	<b>SEX</b>	<b>MEDIAN AGE (YEARS)</b>	<b>RANGE (YEARS)</b>
Normal	tibia, femur	1	F	66	
Normal	tibia, femur	3	M	70	61 - 78
OA	tibia, femur	1	F	66	
OA	tibia, femur	3	M	70	61 - 78

**Tissues used in western blotting to detect threonine phosphorylated proteins**

<b>TYPE OF ARTICULAR CARTILAGE</b>	<b>SOURCE</b>	<b>NUMBER</b>	<b>SEX</b>	<b>MEDIAN AGE (YEARS)</b>	<b>RANGE (YEARS)</b>
Normal	tibia, femur	2	F	70	65 - 74
Normal	tibia, femur	3	M	64	50 - 77

### **Tissues used in immunoprecipitation**

<b>TYPE OF ARTICULAR CARTILAGE</b>	<b>SOURCE</b>	<b>NUMBER</b>	<b>SEX</b>	<b>MEDIAN AGE (YEARS)</b>	<b>RANGE (YEARS)</b>
Normal	tibia, femur	4	F	74	59 - 89
Normal	tibia, femur	2	M	74	

### **Tissues used in immunofluorescent localisation of NG2/HMPG**

<b>TYPE OF ARTICULAR CARTILAGE</b>	<b>SOURCE</b>	<b>NUMBER</b>	<b>SEX</b>	<b>MEDIAN AGE (YEARS)</b>	<b>RANGE (YEARS)</b>
Normal	tibia, femur	6	F	72	59 - 84
Normal	tibia, femur	8	M	65	40 - 90
OA	tibia, femur	2	F	60	59 - 61
OA	tibia, femur	3	M	69	61 - 77

### **Tissues used in immunofluorescent localisation of NG2/HMPG and ECM proteins FN, CII and CVI**

<b>TYPE OF ARTICULAR CARTILAGE</b>	<b>SOURCE</b>	<b>NUMBER</b>	<b>SEX</b>	<b>MEDIAN AGE (YEARS)</b>	<b>RANGE (YEARS)</b>
Normal	tibia, femur	2	F	76	67 - 84
Normal	tibia, femur	1	M	82	

**Tissues used in immunofluorescent localisation of NG2/HMPG and actin**

TYPE OF ARTICULAR CARTILAGE	SOURCE	NUMBER	SEX	MEDIAN AGE (YEARS)	RANGE (YEARS)
Normal	tibia, femur	1	F	76	
Normal	tibia, femur	3	M	59	40 - 77

**Tissues used in immunofluorescent localisation of NG2/HMPG and PDGF $\alpha$ -R**

TYPE OF ARTICULAR CARTILAGE	SOURCE	NUMBER	SEX	MEDIAN AGE (YEARS)	RANGE (YEARS)
Normal	tibia, femur	2	F	65	61 - 69
Normal	tibia, femur	1	M	61	

**Tissues used in cell adhesion assays to study chondrocyte adhesion to FN, CII and CVI**

TYPE OF ARTICULAR CARTILAGE	SOURCE	NUMBER	SEX	MEDIAN AGE (YEARS)	RANGE (YEARS)
Normal	tibia, femur	2	F	71	65 - 77
Normal	tibia, femur	2	M	67	58 - 76
OA	tibia, femur	3	F	68	65 - 71
OA	tibia, femur	1	M	58	

**Tissues used in cell adhesion assays to study the effect of anti- $\beta$ 1 and HMPG antibodies on chondrocyte adhesion to FN, CII and CVI**

TYPE OF ARTICULAR CARTILAGE	SOURCE	NUMBER	SEX	MEDIAN AGE (YEARS)	RANGE (YEARS)
Normal	tibia, femur	1	F	77	
Normal	tibia, femur	2	M	71	65 - 77
OA	tibia, femur	1	F	77	
OA	tibia, femur	2	M	57	49 - 65

**Tissues used in cell adhesion assays to study the effect of increasing doses of M28B5 on chondrocyte adhesion to CVI**

TYPE OF ARTICULAR CARTILAGE	SOURCE	NUMBER	SEX	MEDIAN AGE (YEARS)	RANGE (YEARS)
Normal	tibia, femur	1	F	74	
Normal	tibia, femur	2	M	67	66 - 68

**Tissues used in cell adhesion assays to study the effect of increasing doses of UCHT-1 on chondrocyte adhesion to CVI**

TYPE OF ARTICULAR CARTILAGE	SOURCE	NUMBER	SEX	MEDIAN AGE (YEARS)	RANGE (YEARS)
Normal	tibia, femur	1	M	68	

**Tissues used in cell adhesion assays to study the effect of inhibitors of intracellular signalling molecules on chondrocyte adhesion to CVI**

<b>TYPE OF ARTICULAR CARTILAGE</b>	<b>SOURCE</b>	<b>NUMBER</b>	<b>SEX</b>	<b>MEDIAN AGE (YEARS)</b>	<b>RANGE (YEARS)</b>
Normal	tibia, femur	1	F	89	
Normal	tibia, femur	2	M	67	66 - 68

**Tissues used in western blotting to detect tyrosine phosphorylated proteins**

<b>TYPE OF ARTICULAR CARTILAGE</b>	<b>SOURCE</b>	<b>NUMBER</b>	<b>SEX</b>	<b>MEDIAN AGE (YEARS)</b>	<b>RANGE (YEARS)</b>
Normal	tibia, femur	1	F	75	
Normal	tibia, femur	2	M	55	52 - 60

## APPENDIX IV

Raw data

**Comparison of expression of NG2/HMPG in normal and OA cartilage by densitometry of western blots (Band intensity)**

	EXP. 1	EXP. 2	EXP. 3	EXP. 4	MEAN	SEM
CSPG N	100	100	100	100	<b>100</b>	0
CSPG OA	56	56	42	50	<b>51</b>	4.7
CORE – cABC N	100	100	100	100	<b>100</b>	0
CORE – cABC OA	40	43	36	41	<b>40</b>	2
CORE +cABC N	100	100	100	100	<b>100</b>	0
CORE +cABC OA	41	47	43	46	<b>44</b>	1.8

**Comparison of expression of FN in normal and OA cartilage by densitometry of western blots (Band intensity)**

	EXP. 1	EXP. 2	EXP. 3	EXP. 4	MEAN	SEM
FN N	56	31	17	20	<b>31</b>	8.85
FN OA	100	100	100	100	<b>100</b>	0

**Normal chondrocyte adhesion to FN, CII and CVI (Relative adhesion)**

Substrate	EXP. 1	EXP. 2	EXP. 3	MEAN	SEM
BSA	100	100	100	<b>100</b>	0
FN	542	303	1853	<b>899</b>	481.8
CII	587	323	2100	<b>1003</b>	553.6
CVI	595	263	763	<b>540</b>	146.9



**OA Chondrocyte adhesion to BSA, FN, CII and CVI (Relative adhesion)**

Substrate	EXP. 1	EXP. 2	EXP. 3	MEAN	SEM
BSA	100	100	100	<b>100</b>	0
FN	908	611	1407	<b>975</b>	232.2
CII	1019	623	1425	<b>1022</b>	231.5
CVI	929	377	748	<b>685</b>	162.4

**Effect of anti  $\beta 1$  integrin and HMPG antibodies on the adhesion of chondrocytes isolated from normal cartilage to FN (Relative adhesion)**

Substrate	EXP. 1	EXP. 2	EXP. 3	MEAN	SEM
No antibody	100	100	100	<b>100</b>	0
P4C10	48	52	41	<b>47</b>	3.2
TS2/16	83	151	86	<b>107</b>	22.1
M28B5	86	149	103	<b>113</b>	18.8

**Effect of anti  $\beta 1$  integrin and HMPG antibodies on the adhesion of chondrocytes isolated from normal cartilage to CII (Relative adhesion)**

Substrate	EXP. 1	EXP. 2	EXP. 3	MEAN	SEM
No antibody	100	100	100	<b>100</b>	0
P4C10	47	53	23	<b>41</b>	9.2
TS2/16	87	154	81	<b>107</b>	23.4
M28B5	101	145	100	<b>115</b>	14.8

**Effect of anti  $\beta 1$  integrin and HMPG antibodies on the adhesion of chondrocytes isolated from normal cartilage to CVI (Relative adhesion)**

Substrate	EXP. 1	EXP. 2	EXP. 3	MEAN	SEM
No antibody	100	100	100	<b>100</b>	0
P4C10	55	37	25	<b>39</b>	8.7
TS2/16	101	163	141	<b>135</b>	18.1
M28B5	104	119	118	<b>114</b>	1.5

**Effect of anti  $\beta$ 1 integrin and HMPG antibodies on the adhesion of chondrocytes isolated from OA cartilage to FN (Relative adhesion)**

Substrate	EXP. 1	EXP. 2	EXP. 3	MEAN	SEM
No antibody	100	100	100	<b>100</b>	0
P4C10	63	64	62	<b>63</b>	0.58
TS2/16	101	115	97	<b>104</b>	5.5
M28B5	93	136	109	<b>113</b>	12.5

**Effect of anti  $\beta$ 1 integrin and HMPG antibodies on the adhesion of chondrocytes isolated from OA cartilage to CII (Relative adhesion)**

Substrate	EXP. 1	EXP. 2	EXP. 3	MEAN	SEM
No antibody	100	100	100	<b>100</b>	0
P4C10	9	56	40	<b>35</b>	13.8
TS2/16	88	104	120	<b>104</b>	9.2
M28B5	80	117	147	<b>115</b>	19.4

**Effect of anti  $\beta$ 1 integrin and HMPG antibodies on the adhesion of chondrocytes isolated from OA cartilage to CVI (Relative adhesion)**

Substrate	EXP. 1	EXP. 2	EXP. 3	MEAN	SEM
No antibody	100	100	100	<b>100</b>	0
P4C10	32	41	49	<b>41</b>	4.9
TS2/16	98	177	125	<b>133</b>	23.2
M28B5	85	136	157	<b>126</b>	21.4

**Effect of increasing concentrations of M28B5 and UCHT-1 on chondrocyte adhesion to CVI (Relative adhesion)**

<b>Conc.</b> <b>(µg/ml)</b>	<b>M28B5</b> <b>EXP. 1</b>	<b>M28B5</b> <b>EXP. 2</b>	<b>M28B5</b> <b>EXP. 3</b>	<b>M28B5</b> <b>MEAN</b>	<b>M28B5</b> <b>SEM</b>	<b>UCHT1</b> <b>EXP. 1</b>
0	100	100	100	<b>100</b>	0	100
1	259	132	186	<b>192</b>	36.8	102
10	263	145	218	<b>209</b>	34.4	106
50	318	173	225	<b>230</b>	42.4	111
100	370	179	243	<b>264</b>	56.1	108
500	456	188	290	<b>311</b>	78.1	98

**Effect of inhibitors of intracellular signalling molecules on chondrocyte adhesion to CVI (Relative adhesion)**

<b>Substrate</b>	<b>EXP. 1</b>	<b>EXP. 2</b>	<b>EXP. 3</b>	<b>MEAN</b>	<b>SEM</b>
BSA	100	100	100	<b>100</b>	0
CVI	691	696	404	<b>597</b>	97
CVI+M28B5	1940	1476	1394	<b>1603</b>	170
CVI+gen	832	624	492	<b>649</b>	99
CVI+M28B5+gen	1921	1461	1586	<b>1656</b>	137
CVI+st	483	767	447	<b>566</b>	101
CVI+M28B5+st	1755	1285	1348	<b>1463</b>	147
CVI+cd	370	135	191	<b>232</b>	71
CVI+M28B5+cd	1075	130	575	<b>593</b>	141
CVI+pt	590	663	405	<b>553</b>	77
CVI+M28B5+pt	957	435	894	<b>762</b>	165

**Publications:**

Midwood KS and Salter DM. (abstract) NG2 Proteoglycan is expressed in human adult articular chondrocytes. *International Journal of Experimental Pathology* 1996; 77:6 A28-45.

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Midwood KS and Salter DM. (abstract) NG2/HMPG modulates human articular chondrocyte interaction with type VI collagen. *Journal of Pathology* 1999; (in press).

Midwood KS and Salter DM Modulation of human articular chondrocyte interaction with type VI collagen by NG2/HMPG. *Osteoarthritis and Cartilage* (submitted Jan 1999).